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Prof. M. SYMANN
Oncology Unit
Faculty of Medicine, UCL, Brussels

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CURRICULUM VITAE

1. PERSONAL INFORMATION

NAME	:	SYMANN Michel
BIRTHDATE	:	
PLACE OF BIRTH	:	REDACTED
CITIZENSHIP	:	
HOME ADDRESS	:	
MARITAL STATUS	:	REDACTED

2. EDUCATION

HIGH SCHOOL EDUCATION

1954 - 1960	:	Humanités Greco-Latines Collège Saint Michel, Brussels

UNIVERSITY EDUCATION

1960 - 1967	:	Studies towards the degree of Medical Doctor, Catholic University of Louvain. Degree obtained in June 1967 with Magna Cum Laude.

POST-GRADUATE TRAINING

October 1971 - July 1973	:	Hematology Research Fellow in the laboratory of Professor Frederick Stohlman Jr., Tufts University, Brighton, Massachusetts, 02135, U.S.A.
October 1970 - September 1971	:	Foreign Resident Dóctor in the Department of Professor J. Bernard, Hôpital Saint-Louis, Place du Docteur Fournier, 2 Paris X, France
August 1967 - September 1970	:	Full-time Resident in the Department of Internal Medicine, Professor F. Lavenne, Cliniques Universitaires St Pierre, Louvain, Belgium

POST-GRADUATE EXAMINATION

1979	:	Agrégé de l'Enseignement Supérieur (Ph.D.)
1970 (May)	:	Certification Examination in Internal Medicine
1970 (February)	:	E.C.F.M.G. - written successfully
1967	:	Competition for the admission to the Department of Internal Medicine organized by the Commission of Internal Medicine, Catholic University of Louvain. Placed 3rd out of 34 candidates.

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3. ACADEMIC/PROFESSIONAL APPOINTMENTS

October 1990 - present	:	Professor of Medicine Catholic University of Louvain, Brussels, Belgium
June 1988 - present	:	Head, Unité Facultaire d'Oncologie (Laboratory of Experimental Oncology and Hematology), Catholic University of Louvain
September 1981 - October 1990	:	Associate Professor of Medicine Chargé de Cours Extraordinaire Catholic University of Louvain, Brussels, Belgium
October 1978 - December 1988	:	Medical Director Ludwig Insitute for Cancer Research (Brussels Branch), Avenue Hippocrate, 74, UCL 7459, 1200 Brussels
October 1976 - September 1981	:	Chef de Travaux Unité de Recherche sur les Maladies du Sang Catholic University of Louvain
August 1973 - October 1976	:	Premier Assistant Unité de Recherche sur les Maladies du Sang, Catholic University of Louvain
July 1972 - July 1973	:	Instructor in Medicine Tufts School of Medicine, Boston, Massachusetts,02111, U.S.A.

4. HOSPITAL APPOINTMENTS

October 1988 - present	:	Head Clinical Oncology Department St Luc University Hospital;
October 1984 - September 1988	:	Chef de Clinique Clinical Oncology Unit, St Luc University Hospital, University Hospital, Avenue Hippocrate 10, 1200 Brussels
December 1983 - September 1984	:	Chef de Clinique Associé Hematology and Oncology Units, St. Luc University Hospital
August 1976 - November 1983	:	Chef de Clinique Adjoint Hematology Unit, St Luc University Hospital

5. UNIVERSITY/HOSPITAL COMMITTEES

1984 - 1988	:	Groupe de travail, Hospitalisation de Jour, Conseil Communautaire des Etablissements de soins, Ministère de la Communauté Française
1980 - 1982	:	Conseil de Recherche, Catholic University of Louvain

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6. SCIENTIFIC - ADMINISTRATIVE RESPONSABILITIES

1989 - present	:	Secrétaire Général, Oeuvre Belge du Cancer 21 Rue des Deux Eglises, 1040 Bruxelles
1988 - present	:	Member of the Advisory Committee, International Autologous Bone Marrow Transplantation Registry
1988 - 1990	:	Member of the Scientific Committee of the "Association contre le Cancer", Place du Samedi 13, Bte 13, 1000 Bruxelles
1988 - present	:	Member of the "Comité Scientifique de l'Institut d'Hématologie", Université Paris VII, Hôpital Saint-Louis, 2 Place du Docteur-Fournier, 75475 Paris Cedex 10
1987 - present	:	Member of the Scientific Committee of "Eurocancer"
1987 - present	:	Member of the "Comité de Coordination dans le domaine du cancer", INSERM (France)
1986 - present	:	Administrateur, Oeuvre Belge du Cancer 21 Rue des Deux Eglises, 1040 Bruxelles
1985 - present	:	Member of the Scientific Committee of the "European School of Haematology"
1976 - 1988	:	Head, Laboratoire d'Hématologie Expérimentale, Catholic University of Louvain

7. HONORS

1991	:	Founding Member of the European Hematology Association
1984 - 1992	:	Chairman, E.O.R.T.C. Autologous Bone Marrow Transplantation Study Group
1984 - 1985	:	President of the "Groupe Prolifération et différenciation cellulaire", Société Française d'Hématologie
1983 - 1988	:	Member of the International Institute of Cellular and Molecular Pathology (Brussels)
1972 - 1973	:	Public Health Service International Fellowship (National Institutes of Health, U.S.A.)
1971 - 1972	:	Damon Runyon Memorial Cancer Research Fellowship (U.S.A.)
1970 - 1971	:	Fellow of the "Collège de Médecine des Hopitaux de Paris"

8. EDITORIAL BOARD

Experimental Hematology

Focus on Growth Factors

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9. SCIENTIFIC SOCIETY MEMBERSHIPS

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10. INVITED SPEAKER AT THE UNIVERSITY

ULB (Belgique), Paris (France), Friburg (Allemagne), Harvard (USA), VUB (Belgique), Liège (Belgique), Lyon (France), Grenade (Espagne), Charlottesville (USA), Nebraska (USA), Berlin (Allemagne), Besançon (France), KUL (Belgique)

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PUBLICATIONS

1. SOKAL G, SYMANN M.
Hémoglobinurie de marche chez une jeune fille.
Nouv Rev Fr Hématol 1972; 12:901-906.

2. STOHLMAN F Jr, QUESENBERRY P, NISKANEN E, MORLEY A, TYLER W, RICHARD K, SYMANN M, MONETTE F, HOWARD D.
Control of granulopoiesis - in Haemopoietic stem cells.
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3. QUESENBERRY P, NISKANEN E, SYMANN M, HOWARD D, RYAN M, HALPERN J, STOHLMAN F Jr.
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Cell Tissue Kinetics 1974; 7:337-348.

4. NISKANEN E, TYLER WS, SYMANN M, STOHLMAN F Jr, HOWARD D.
The effect of neutropenia on the cell cycle of granulocyte precursors in an in vivo culture system.
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5. TYLER WS, NISKANEN E, SYMANN M, STOHLMAN F Jr.
Studies on myelopoiesis and stem cells : proliferation in in vivo culture system.
In ROBINSON WA, ed. Hemopoiesis in culture : Sponsored by the National Cancer Institute, 1974;171-179. (DHEW Publication N° (NIH)74-205).

6. SYMANN M.
Anémies macrocytaires.
Louvain Med 1975; 94:225-226.

7. SYMANN M.
Virus et leucémies humaines.
Documents de Synthèse, Association Royale de la Presse Médicale Belge 1975.

8. SYMANN M, FONTEBUONI A, QUESENBERRY P, HOWARD D, STOHLMAN F Jr.
Fetal hemopoiesis in diffusion chamber cultures : I. The pattern of pluripotent stem cell growth.
Cell Tissue Kinetics 1976; 9:41-49.

9. SYMANN M, QUESENBERRY P, FONTEBUONI A, STOHLMAN F Jr.
Fetal hemopoiesis in diffusion chamber cultures : II. Cell proliferation and differentiation.
Nouv Rev Fr Hématol 1976; 16:321-328.

10. SYMANN M, QUESENBERRY P, FONTEBUONI A, HOWARD D, RYAN A, STOHLMAN F Jr.
Fetal hemopoiesis in diffusion chamber cultures : III. The effect of neutropenia.
Blood 1976; 48:283-291.

11. SYMANN M, RODHAIN J, HUMBLET Y.
La greffe de moelle osseuse humaine allogénique.
Louvain Med 1977; 96:115-120.

12. SYMANN M, ANCKAERT MA, CORDIER A, RODHAIN J, SOKAL G.
Murine yolk sac hematopoiesis studied with the diffusion chamber technique.
Exp Hematol 1978; 6:749-759.

13. SYMANN M.
Fetal hemopoiesis in diffusion chamber cultures.
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14. HUYBRECHTS M, SYMANN M, TROUET A.
Effects of daunorubicin and doxorubicin, free and associated with DNA on hemopoietic stem cells.
Cancer Res 1979; 39:3738-3743.

15. HUYBRECHTS M, SYMANN M, TROUET A.
The diffusion chamber technique as an in vivo assay in mice for the effectiveness of antitumor agents.
Scand J Haematol 1979; 23:223-226.

16. HUYBRECHTS M, SYMANN M.
La culture de moelle dans les leucémies et les cytopénies.
Documents de Synthèse, Association Royale de la Presse Médicale Belge 1980; 108-112.

17. QUESENBERRY P, SULLIVAN R, FONTEBUONI A, LEVITT L, NISKANEN E, SYMANN M, MONETTE FC, ZUCKERMAN A, RYAN M.
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Berlin: Springer Verlag, 1980; 44-53.

18. HUYBRECHTS M, TROUET A, SYMANN M.
Activity in vivo of daunorubicin assayed by the diffusion chamber technique.
Nouv Rev Fr Hématol 1980; 22:1-5.

19. SYMANN M, RODHAIN J, HUYBRECHTS M, CHATELAIN C, HUMBLET Y, CANON JL, SOKAL G.
In vivo studies of a murine myeloblastic leukemia.
Nouv Rev Fr Hématol 1980; 22:63-68.

20. SYMANN M, ANKAERT MA, QUESENBERRY P, FONTEBUONI A, RODHAIN J, CHATELAIN C, HUYBRECHTS M, SOKAL G.
Fetal stem cells in diffusion chamber cultures.
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Amsterdam: Exc Med 1980 ; 19-28.

21. SYMANN M, HUYBRECHTS M, CHATELAIN C, CANON JL, RODHAIN J, SOKAL G.
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Nouv Rev Fr Hématol 1980; 22 (Suppl):17-24.

22. FERRANT A, RODHAIN J, CORDIER A, SYMANN M, MICHAUX JL, SOKAL G.
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Scand J Haematol 1980; 25:12-18.

23. HULHOVEN R, MICHAUX JL, CORNU G, FERRANT A, SYMANN M, BOSLY A, DELANNOY A, DUTRIEUX-FAUCHET MC, SOKAL G.
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24. SYMANN M, DE MONTPELLIER C, NINANE J, VAN DEN BERGHE H.
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Cancer Gen and Cytogen 1982; 6:183-185.

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25. SYMANN M, NINANE J, ANCKAERT MA, HUYBRECHTS M, SOKAL G.
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Exp Hematol Today 1982; 31-36.

26. SYMANN M, ANCKAERT MA, HUYBRECHTS M, NINANE J, CANON JL, SOKAL G.
In vivo stimulation and inhibition of granulopoiesis. The effect of an inflammatory reaction on murine diffusion chamber granulopoiesis.
Brit J Haematol 1982; 51:89-98.

27. DE MONTPELLIER C, CORNU G, RODHAIN J, SOKAL G, SYMANN M.
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Blood Cells 1982; 8:439-444.

28. SYMANN M.
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Louvain Med 1982; 101:255-257.

29. SYMANN M, BOSLY A.
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Louvain Med 1982; 101: 258-260.

30. ANCKAERT MA, SYMANN M.
In vivo induction of granulopoiesis in visceral yolk-sac cells by foetal hepatic factors.
J Embryol Exp Morphol 1983; 73:87-95.

31. NINANE J, CANON JL, CORNU G, SOKAL G, SYMANN M.
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Int J Cell Cloning 1984; 2:216-226.

32. SYMANN M, LEBACQ AM, GORIN NC, HUMBLET Y, LOPEZ M, DOUAY L, BOSLY A, LAPORTE JP, NINANE J, SALMON Ch, BAZIN H, NAJMAN A, CORNU G, DUHAMEL G, SOKAL G.
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In SPITZY KH, KARRER K, eds. Proceedings of the 13th International Congress of Chemotherapy. Vienna: SS 76-11, 1983; 232:38-42.

33. SYMANN M, BOSLY A, HUMBLET Y, DELAUNOIS L, STEYAERT J, FRANCIS C, MACHIELS J, PRIGNOT J.
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In SPITZY KH, KARRER K, eds. Proceedings of the 13th International Congress of Chemotherapy. Vienna: SS 76-7,1983; 232:20-24.

34. MECUCCI C, MICHAUX JL, BROECKAERT VAN ORSHOVEN A, SYMANN M, BOOGAERTS M, KULLING G, VAN DEN BERGHE H. Translocation t(3;17)(q26;q22): A marker of acute disease in myeloproliferative disorders ?
Cancer Gen and Cytogen 1984; 12:111-119.

35. HUMBLET Y, SYMANN M, BOSLY A, DELAUNOIS L, STEYAERT J, FRANCIS C, MACHIELS J, PRIGNOT J.
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36. LEBACQ-VERHEYDEN AM, HUMBLET Y, RAVOET AM, SYMANN M.
Immunological removal of cancer cells in bone marrow autografts : Setting the experimental conditions.
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37. WILLARD KE, HUMBLET Y, SYMANN M.
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38. MECUCCI C, SYMANN M, BOSLY A, TRICOT G, VAN DEN BERGHE H.
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39. LEBACQ-VERHEYDEN AM, HUMBLET Y, NEIRYNCK A, RAVOET A, SYMANN M.
Four rat cytotoxic monoclonal antibodies for the in vitro treatment of bone marrow autografts in non-T non-B acute lymphoblastic leukemias. In DICKE KA, SPITZER G, ZANDER A, eds. Autologous bone marrow transplantation. Proceedings of the First International Symposium. Houston, Texas: University of Texas M.D. Anderson Hospital and Tumor Institute, 1985;419-423.

40. SYMANN M, HUMBLET Y, BOSLY A, DELAUNOIS L, FRANCIS C, PRIGNOT J.
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In DICKE KA, SPITZER G, ZANDER A, eds. Autologous bone marrow transplantation. Proceedings of the First International Symposium. Houston, Texas: University of Texas M.D. Anderson Hospital and Tumor Institute, 1985;161-165.

41. SYMANN M, NINANE J, HAMOOD M, CHATELAIN C, CANON JL, SOKAL G.
In vivo stimulation and inhibition of granulopoiesis at the stem cell level. In CRONKITE E, ed. Stem cell physiology. New York: Alan R. Liss, 1985;203-212.

42. SYMANN M, CHATELAIN C.
Physiopathologie de la maladie de Vaquez.
In NAJEAN Y, ed. Les Polyglobulies. Paris: Masson, 1985; 15-34.

43. SYMANN M, CHATELAIN C, HUMBLET Y.
Réunion conjointe des Groupes d'Études sur "La prolifération et la différenciation cellulaire" et "l'Hémostase et la thrombose". Bruxelles, 26-27 octobre 1984.
Nouv Rev Fr Hématol 1985; 27:333-346.

44. GHIONE F, MECUCCI C, SYMANN M, MICHAUX JL, CASTEELS-VAN DAELE M, VAN DEN BERGHE H.
Cytogenetic investigations in childhood chronic myelocytic leukemia.
Cancer Gen and Cytogen 1986; 20:317-323.

45. CEULEMANS F, HUMBLET Y, BOSLY A, SYMANN M, TROUET A.
A phase I study of vinblastine tryptophan ester.
Cancer Chemoth and Pharmacol 1986; 18:44-46.

46. COIFFIER B, BOSLY A, CALIGARIS-CAPPIO F, GISSELBRECHT C, PATTE C, SCHAADT M, SYMANN M.
Management of Non-Hodgkin's Lymphomas : Conclusions of the European School of Oncology meeting 1986. Eur J Cancer Clin Oncol 1987; 23:1691-1695.

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47. SYMANN M, HUMBLET Y, CANON JL, LEBACQ-VERHEYDEN AM.
Detection of bone marrow metastases in small cell lung cancer by tumor stem cell assay and by monoclonal antibodies.
In Dicke KA, Spitzer G, Jagannath S, eds: Autologous bone marrow transplantation. Proceedings of the Third International Symposium. Houston, Texas: University of Texas M.D. Anderson Hospital and Tumor Institute, 1986;509-513.

48. BOSLY A, SYMANN M.
Effect of recombinant human interleukin 2 on T-L CFU after autologous bone marrow transplantation : Preliminary Results.
In Dicke KA, Spitzer G, Jagannath S, eds: Autologous bone marrow transplantation. Proceedings of the Third International Symposium. Houston, Texas: University of Texas M.D. Anderson Hospital and Tumor Institute, 1986;697-703.

49. HUMBLET Y, SYMANN M, BOSLY A, DELAUNOIS L, FRANCIS C, MACHIELS J, BEAUDUIN M, DOYEN C, WEYNANTS P, J LONGUEVILLE, PRIGNOT J.
Late intensification chemotherapy with autologous bone marrow transplantation in selected small cell carcinoma of the lung : A randomized study.
J Clin Oncol 1987; 5:1864-1873.

50. HUMBLET Y, SYMANN M.
Clinical Studies of high dose therapy plus autologous bone marrow transplantation in small cell lung cancer.
Bone Marrow Transplantation 1987; 2(Suppl 1):194-196.

51. BOSLY A, STAQUET Ph, DOYEN C, CHATELAIN B, HUMBLET Y, SYMANN M.
Recombinant human Interleukin-2 restores in vitro T cell colony formation by peripheral blood mononuclear cells after autologous bone marrow transplantation.
Exp Hematol 1987; 15:1048-1054.

52. CHATELAIN C, DE BAST M, SYMANN M.
Identification of a light density murine megakaryocyte progenitor.
Blood 1988; 72:1187-1192.

53. HUMBLET Y, CANON JL, SEKHAVAT M, FEYENS AM, MANOUVRIEZ P, LEBACQ-VERHEYDEN AM, BAZIN H, PRIGNOT J, SYMANN M.
Detection of small cell lung cancer bone marrow metastases by immunofluorescence.
Path Biol 1988; 36:25-28.

54. HUMBLET Y, SYMANN M.
Detection of small cell lung cancer bone marrow metastases by tumor stem cell assay.
Path Biol 1988; 36:83-85.

55. CANON JL, HUMBLET Y, LEBACQ-VERHEYDEN AM, MANOUVRIEZ P, BAZIN H, RODHAIN J, PRIGNOT J, SYMANN M.
Immunodetection of small cell lung cancer metastases in bone marrow using three monoclonal antibodies.
Eur J Cancer Clin Oncol 1988; 24:147-150.

56. LEBACQ-VERHEYDEN AM, NEIRIJNCK A, RAVOET AM, HUMBLET Y, OIE H, LINNOILA I, GAZDAR A, MINNA J, SYMANN M.
Monoclonal antibodies for the in vitro detection of small cell lung cancer metastases in human bone marrow.
Eur J Cancer Clin Oncol 1988; 24:137-145.

57. HUMBLET Y, SEKHAVAT M, FEYENS AM, SYMANN M.
Cytotoxicity of Workshop Monoclonal Antibodies in the Presence of Complement. Lung Cancer 1988; 4:90-91.

58. JACQUES CH, LEFEVRE PH, DEWITTE A, NGUYEN R, HUMBLET Y, LONGUEVILLE J, SYMANN M.
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59. HUMBLET Y, LEFEBVRE P, JACQUES HL, BOSLY A, FEYENS AM, SEKHAVAT M, AGALIOTIS D, SYMANN M.
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60. WEYNANTS P, WAUTERS P, COULIE PG, VAN DEN EYNDE B, SYMANN M, BOON T.
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61. LONGUEVILLE J, HUMBLET Y, SYMANN M.
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62. SYMANN M, CHATELAIN C, AGALIOTIS D.
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64. SYMANN M, BOSLY A, GISSELBRECHT C, BRICE P, FRANKS C.
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65. WEYNANTS P, HUMBLET Y, BOSLY A, SCHALLIER D, DUPREZ P, MAJOIS F, BEAUDUIN M, PRIGNOT J, SYMANN M.
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66. CHATELAIN C, HAMOOD M, DE BAST M, SYMANN M.
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67. HUMBLET Y, FEYENS AM, SEKHAVAT M, AGALIOTIS D, CANON JL, SYMANN M.
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70. SYMANN M, HUMBLET Y, BOSLY A, CANON JL, DOYEN C, LONGUEVILLE J.
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71. FRERE AM, HUMBLET-KOCH MC, HUMBLET Y, LONGUEVILLE J, SYMANN M.
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Louvain Med 1989; 108:345-354.

72. NISKANEN E, CHATELAIN C, SYMANN M.
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Int J Cell Cloning 1989 ; 7 (6): 330-342.

73. SHILONI E, POUILLART P, JANSSENS J, SPLINTER T, DI PERI T, SYMANN M, ROEST GJ, PALMER PA, FRANKS CR.
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TAMOXIFEN/HDMPA

RESUME DU PROTOCOLE (appliqué à St. Luc)

Comparaison du Tamoxifen et de la Médroxyprogestérone chez les patientes pré- ou postménauposées, sans envahissement ganglionnaire et sans métastases à distance.

Le Taxomifen sera prescrit pendant 5 ans à raison de 20 mg/j tandis que la Médroxyprogestérone sera prescrite pendant 9 mois à raison de 1g per os par jour.

OBJECTIFS

- Comparer l'activité dans le cancer du sein sans envahissement ganglionnaire du traitement adjuvant Tamoxifen (TAM), à l'acétate de Médroxyprogestérone (HD MPA), évaluée par l'intervalle libre et la survie.
- Comparer la tolérance des 2 traitements.
- (Etude de nouveaux facteurs pronostic cancer du sein : Cathepsine D, % --- en phase S, oncogène Neu et sa protéine, EGFR)

DEFINITION DU STATUS POST-MENOPAUSAL

Est considérée comme ménopausée la patiente dont les dernières menstruations remontent à plus d'un an. Lorsque la patiente a subi une hystérectomie sans ovariectomie, elle est considérée comme ménopausée à l'âge de 50 ans ou plus. En outre, est considérée comme ménopausée une patiente ayant subi une ovariectomie bilatérale.

CRITERES D'INCLUSION

1. La patiente a moins de 70 ans
2. La tumeur est un cancer du sein histologiquement prouvé et opéré d'emblée.
3. La tumeur est du stade T1c, T2, T3 ou T4 opérable
4. La tumeur est histologiquement bien ou moyennement différenciée (SBRI ou II) en préménopause et également SBRIII en post-ménopausé.
5. Au moins 8 ganglions ont été examinés et aucun n'est envahi histologiquement.
6. Il y a absence de métastases selon le bilan complet préalable à la randomisation.
7. Le performance status est 0 ou 1.
8. Il y a accord de la patiente après information, selon les usages du Centre.

CRITERES D'EXCLUSION

1. La tumeur est du stade T1s, T1a, T1b.
2. La tumeur est du stade T4 non opérable.
3. La patiente est en pré-ménopause avec tumeur mal différenciée SBRIII.
4. La patiente présente une mastite carcinomateuse.
5. La patiente est enceinte.
6. La patiente présente des troubles hématologiques (neutrophiles < 2000/mm^3 ou globules blancs < 3500/mm^3 ou plaquettes sanguines < 200000/mm^3).
7. La patiente présente des troubles hépatiques (bilirubine directe > 1,2 mg/100ml).
8. La patiente présente des troubles rénaux (créatinine > 1,5 mg/100 ml).
9. La patiente a des antécédents de troubles thrombo-emboliques sévères ou cardiaques (insuffisance cardiaque, coronaire ou HTA non contrôlée).

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10. La patiente présente des troubles neurologiques ou autres pouvant rendre un suivi correct difficile.
11. La patiente a été traitée par des progestogènes les 6 derniers mois précédant l'inclusion de l'étude, à l'exclusion d'un contraceptif oral.
12. La patiente a un autre cancer simultané, à l'exception d'un cancer in situ du col de l'utérus et d'un épithélioma de la peau traité.
13. La patiente présente un cancer du sein controlatéral.

BILAN PREALABLE A LA RANDOMISATION

En préopératoire : bilan d'extension complet

1. Stadification clinique TNM
2. Mammographie
3. Marqueurs tumoraux CA 15.3, CEA
4. Thorax, ECG
5. Biologie :
   - Hémogramme avec formule leucocytaire et réticulocytes
   - Tests hépatiques : bilirubine directe et indirecte, GOT, GPT, gammaGT, phosphatases alcalines.
   - Tests inflammatoires : VS, CRP, ---
   - Ionogramme
   - Urée et créatinine sanguine.
6. Echographie et/ou CT scan hépatique
7. Scintigraphie osseuse + RX si points d'hyperfixation

En postopératoire

1. Grade histologique de la tumeur
2. Examen histo d'au moins 8 ggl tous négatifs)
3. Taux RO, RE.

NB : Si suspicion lésion cérébrale CT scan.

FOLLOW-UP

. Une consultation 3 mois après mise en route d'un traitement adjuvant puis tous les 6 mois.
. Un bilan d'extension complet 1 x/an pendant 5 ans
. > 5 ans : suivi clinique 1x/an + mammographie.

Pour les patientes recevant du MPA, un échantillon de 10 ml de sérum sera prélevé à la fin du 3ème et du 9ème mois de traitement (échantillon congelé à - 20°C).

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CMF/ZOLADEX

ESSAI OUVERT RANDOMISE : ZOLADEX VS CMF COMME TRAITEMENT ADJUVANT DANS LE MANAGEMENT DES GANGLIONS ENVAHIS STADE II CANCER DU SEIN EN PRE/PERIMENOPAUSE CHEZ DES PATIENTES AGEES DE 50 AN OU MOINS.

OBJECTIFS

1. Comparer le "disease-free survival". Des patient pré/péri MNP âgées de 50 ans ou moins qui présentent des glangions envahis (stade II cancer du sein) recevant du Cyclophosphamide, du Methotrexate et du 5-Fluorouracil (CMF) ou du Zoladex comme traitement adjuvant.

2. Comparer "Overall Survival" de ces patientes dans les 2 groupes de traitement.

3. Comparer les effets secondaires dans les 2 groupes traitement.

SCHEMAS

- ZOLADEX : 1 implant SC 3.6 mg tous les 28 jours pendant 2 ans.
- CMF : Cyclophosphamide : 500 mg/m^2 I.V. au J1 et 8 cycle 28 J. ou per os J1 au J14.
  - Methotrexate : 40 mg/m^2 I.V. au J1 et 8 cycle 28 jours
  - 5-Fluorouracil : 600 mg/m^2 I.V. au J1 et 8 cycle 28 jours (6 cures)

BILAN PRE-RANDOMISATION

- Examen clinique
- Bilan classique extension pour exclure métas à distance (thorax scinti os on RMN, VX, écho foie ou CT scan si enzymes ---- > bioclassique.

DIAGRAMMATIC REPRESENTATION

SURGERY

PATIENT CONSENT OBTAINED

RANDOMISATION

CMF (6 cycles) — PATIENT REFUSED RANDOMISED THERAPY — ZOLADEX (2 years)

PATIENT FOLLOWED AS DETAILED IN THE PROTOCOL

OR

RECURRENCE — DEATH (without recurrence)

DEATH

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FOLLOW-UP

Rythme :
- toutes les 12 semaines ---> 36 semaines
- à 2 ans
- tous les 6 mois -----> 3 ans
- annuellement --------> 10 ans

Type :
- Examen clinique
- Bio classique
- Autres examens bilan extension

Documenter et préciser la récidive

Indiquer Cause Mort

CRITERES D'INCLUSION

a. Patientes pré/péri ménopausées âgées de 50 ans ou moins
NB : si doute sur status préménopausé ----> dosage FSH

b. Patientes présentant des ganglions envahis stade II cancer du sein
- cancer du sein invasif prouvé histologiquement (T0, T1, T2)
- envahissement --- (N1)
- pas d'évidence de métastases à distance.

c. Pas de traitement systémique préalable pour le cancer du sein

d. Consentement informé des patientes

e. Certains centres dont UCL — Récepteurs oestrogènes + uniquement patientes R - non inclues

CRITERES D'EXCLUSION

a. Ovariectomie bilatérale préalable ou radiothérapie sur les ovaires.

b. Grossesse

c. Deuxième cancer infiltrant à l'exception cancer in situ du col ou cancer -------

d. Randomisation ± 6 semaines après traitement chirurgical.

e.
- 60 < 3000/mm^3
- plaquettes < 100.000/mm^3
- perturbation fonction hépatique
- fonction rénale inadéquate (créatinine sanguine > 1,5 valeurs normales)

f. R0 - (UCL)

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4 EPIADRIAMYCINE HAUTES DOSES + GM-CSF + CYCLOPHOSPHAMIDE

I. RESUME DU PROCOTOLE

. Essai ouvert, incontrôlé visant à déterminer "Maximum Dose Intensity" d'Epirubicine en association avec du Cyclophosphamide, définie comme la quantité de ces drogues/unité de temps qui peut être administrée avec stimulation médullaire par du GM-CSF (permettant diminuer durée nadir neutropénie granulocytes < 200/mm^3 pendant < 5 jours).

. Augmenter alternative des doses de Cyclophosphamide et d'Epirubicin à partir de
300 mg/m^2/W et 60 mg/m^2/W ----> MTD
Une cohorte de 6 patientes est étudiée à chaque niveau de dose. La dose de GM-CSF reste constante tout au long de l'étude.

II. OBJECTIFS

1. Déterminer la "maximal-dose intensity d'Epirubicine en association avec du cyclophosphamide, définie comme la quantité drogues/unité de temps qui peut être administrée avec stimulation médullaire par du GM-CSF (objectif diminuer durée neutropénie < 200/mm^3 < 5 jours).

2. Evaluer le "Safety Profil" d'une chimiothérapie avec intensification de doses association de l'Epirubicine etdu Cyclophosphamide.

3. Evaluer le "Safety Profil" du GM-CSF administré dans cette association.

4. Evaluer l'activité en terme de taux de réponse et de durée de rémission avec ce schéma de chimiothérapie.

CRITERES DE SELECTION

Critères d'inclusion

1. Cancer du sein histologiquement prouvé, métastases à distance (stade IV) ou recurrence locorégionale après mastectomie curage axillaire.

2. Au moins une lésion néoplasique mesurable (uni ou bidimension) par ex. physique et/ou radiologique.

3. Age : entre 18 et 65 ans.

4. Performance status 0,1,2 (selon ECOG)

5. Neutrophiles > 2000 et plaquettes > 100000.

6. Fonction hépatique correcte : bilirubine totale < 1,5 mg/dl et GOT (3x valeurs limites sauf si anomalies GOT attribuées à des métas. hépatiques; fonction rénale normale : créatinine < 1,2 mg/dl ou clearance créatinine > 80 ml/min.

7. Radiothérapie ant. autorisée.

8. Hormonothérapie ant. autorisée.

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9. Absence de toxicité aiguë liée à des traitements antérieurs (chimio ou radiothérapie).

10. LVEF (fraction ejection ventricule gauche) dans les limites si la normale.

11. Consentement informé des patientes.

12. Compliance pour le follow-up.

Critères d'exclusion

1. Récurrence locale après mastectomie partielle.

2. Cancer du sein localisé avancé et inopérable (stade IIIB)

3. Lésions osseuses osteoblastiques comme seule évidence de la maladie.

4. Epanchement pleural et/ou ascite et/ou lymphangite carcinomateuse comme seules manifestations de la maladie.

5. Antécédents de chimiothérapie avec anthracyclines ou anthracenediones.

6. Antécédents de chimiothérapie pour cancer du sein récurrent ou métastatique.

7. Patientes nécessitant une radiothérapie concomitante à titre palliatif.

8. Utilisation des médicaments suivants : corticoïdes à l'exception du contrôle de l'oedème cérébral dû à des métas cérébrales ou pour prophylaxie antiémétique, lithium, chloramphenicol, AINS.

9. Antécédent infarctus du myocarde dans la dernière année ou décompensation cardiaque ou troubles du rythme sévères nécessitant un traitement permanent ou HTA incontrôlée; angor instable.

10. Anomalies suivantes décrites à l'E.C.G. : hypertorphie ventricule gauche (Sokolow > 40), bloc branche G complet, bloc branche D complet + hémibloc ant G ou post G, signes d'insuffisance coronarienne (--- décalage ST), haut risque d'arythmie incontrôlée (extrasystoles ventriculaires multifocales).

11. Autre cancer à l'exception cancer basocellulaire de la peau correctement traité ou canc in situ col utérin.

12. Processus infectieux actif.

13. Evidence clinique ou RX de métastases cérébrales.

14. Grossesse.

15. Pas de traitement concomitant.

CONDUITE DE L'ETUDE

Administration des produits

. Epirubicine et cyclophosphamide sont administrés en i.v. toutes les 2 semaines si les neutrophiles > 2000/mm^3 et les plaquettes > 100000/mm^3. Si ces valeurs ne sont pas atteintes, le traitement est reporté.

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. GM-CSF administré en sous cutané à la dose de 5 μg/kg du J1 au J11.
J1 : leucocytes doivent être < 10.000/mm^3
J12 : si neutrophiles < 2000/mm^3 : traitement par GM-CSF est continué mais dans tous les cas par > 1 semaine

Plan de Traitement

. Une cohorte de 6 patientes est étudiée à chaque niveau de dose.
. Il n'y a pas d'escalade de dose chez une même patiente; une diminution des doses est prévue en cas de myélosuppression ou de toxicité non hémato. sévère.
. Les cures sont administrées au jour prévu (---> neutros > 2000/mm^3 et plaquettes > 100000/mm^3. Si ces valeurs ne sont pas atteintes, la cure est postposée jsuqu'à ce qu'elles soient au niveau requis. L'escalade de dose est autorisée par la cohorte suivante.

si :

a. < 2 patientes/6 montrent les problèmes de toxicité suivants : stomatite grade 3, neutropénie sévère, neutropénie fébrile cardiotoxicité et/ou toute autre toxicité grade 4.

b. < 2 patientes/6 ont > 1 semaine de délai une récupération médullaire (d'au moins 1 des 2 premiers cycles de traitement). A ce moment atteinte MTD si au moins 5 patientes ont une récupération médullaire dans les 3 semaines, l'étude continue au niveau suivant la dose administrée toutes les 3 semaines.

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PLAN OF THE STUDY : IMMUNIZATION OF HLA-A1 PATIENTS CARRYING A BREAST CANCER WHOSE TUMOUR EXPRESSES MAGE-1

1. INTRODUCTION

2. OBJECTIVES OF THE STUDY

3. SELECTION OF PATIENTS

   3.1. Stage of the Disease

   3.2. Inclusion criteria

   3.3. Exclusion criteria

4. THERAPEUTIC SEQUENCE

5. IMMUNIZATION REGIMEN

6. STUDY PARAMETERS

7. IMMUNOLOGICAL STUDIES

8. MECHANICS OF THE STUDY

9. VACCINE SUPPLY

10. ETHICAL CONSIDERATION

11. REFERENCES - APPENDIX

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1. INTRODUCTION

This study is based on the work of researchers at the Ludwig Institute. These researchers have been studying the immunological mecanisms of cancer rejection for a few years. Recently, they have identified a gene that directs the expression of antigen MZ2.E on a human melanoma cell line (MZ2.MEL). This gene called MAGE 1 is expressed by other melanoma cell lines but no expression is observed in normal tissues. Antigen MZ2-E producted by MAGE 1 is recognized by C.T. lymphocytes only if it's presented by HLA. A1. (26% of total in Caucasian population). This human rejection antigen is shared by a significant proportion of other human tumours like breast cancer, small cells lung cancer, soft tissue tumours, respectively in 20%, 40% and 25%. The ability to identify these tumours readily on the basis of their expression of the relevant gene opens new possibilities for specific immunotherapy. Small tumours samples of HLA A1 patients can be frozen rapidly so as to ensure conservation of the RNA. This RNA can be tested by reverse transcription and PCR amplification to identify the tumors that express (gene) MAGE-1. Those tumors expressing antigen MAGE-1 may be sensitive to an CTL response. A study on the melanoma is now in progress. Here we propose a study for breast tumours in order to evaluate the CTL response.

2. OBJECTIVES OF THE STUDY

a) To assess the tolerance of a vaccine made of lethally irradiated allogeneic tumour cells on HLA-A1 patients whose tumour has been found to express MAGE-1.

b) To evaluate wether vaccination with allogeneic cells expressing MAGE-1 and HLA A1 increases the frequency of CTL directed against antigen MZ2-E

3. SELECTION OF PATIENTS

3.1. STAGE OF THE DISEASE

Because the most important aim of this study is to evaluate the CTL response after immunization, we suggest introducing :

a) Node negative premenopausal patients :

. Stage I T1 a ou b N0 M0

b) Node negative post-menopausal patients who are not eligible for protocol Tamoxifène/hautes doses MPA

. Stage I T1a-b N0 M0

. Stage IIA T2 N0 M0, if aged of > 70 ans at the time of diagnosis, not eligible for TMX-HDMPA

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3.2. INCLUSION CRITERIA

To be elected, patients must fullfill all the following criteria :

1. HLA typing HLA.A1 : it is advisable to perform a complete HLA typing

2. Histologically confirmed diagnoses primary breast cancer and belonging to the categories described above

3. Patients should be in a stable general medical condition with performance status according to Karnofsky's scale 100-60, and a life expectancy of 3 months.

4. Age of 80 years or less

5. Patients must be able to comply with scheduled follow-up visit

3.3. EXCLUSION CRITERIA

1. Concomittant chemotherapy

2. Concomittant hormonotherapy

3. Psychosis or neurological alterations

4. History or evidence of allergic or autoimmune disease.

4. THERAPEUTIC SEQUENCE :

- Complete work-up
- Surgery : modified radical mastectomy or lumpectomy and axillary dissection
- Radiotherapy if indicated in all cases of conservative treatment
- Immunotherapy : the first immunization will not be sooner than 30 days after surgery, but may be concommitant of radiotherapy.
- Chemotherapy takes place after irradiation and immunotherapy.

5. IMMUNIZATION REGIMEN

Cells : Breast tumour cell lines will be used. These cell lines will be HLA-A1 and will have high expression of MAGE-1. The patients will therefore be immunized with cells that are allogeneic except for HLA-A1

Time course : Immunization will be carried out on days 0,30,60,120,180,300,420. For those patients who receive surgery, day 0 will not be sooner than 30 days after these treatment. Immunizations may be interrupted at the discretion of the investigator if major recurrence would occur. Most : each immunization will consist injections of 10^7 irradiated cells (one of each above mentioned cell lines). The cells will be irradiated at 10000 rads and will injected intradermally in the inguinal area where lymph drainage is not involved with the tumor site.

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6. STUDY PARAMETERS

. Before the start of therapy : a complete medical history must be taken and a complete physical examination performed. Appropriate work-up must be performed including chest X ray or CT scan, mammography, liver ultrasonography or CT scan, bone scintigraphy, bone X ray .... The extent of the disease will be classified in accordance with the clinical staging system for breast cancer.

. Each patient undergoes a complete biological evaluation

- Haematological tests :
  - WBC
  - platelets
  - Hb
  - Haematocrit
- Special BC. tests :
  - CEA
  - CA 15.3
- Blood chemistry and liver function : Gamma GT, GOT, GPT, alkaline phosphatase, bilirubine
- Follow-up :
  - blood tests = every 6 months
  - extent Rx work-up :
    - every 6 months (the first year)
    - then every year

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GREFFE DE CELLULES SOUCHES HEMATOPOIETIQUES DU SANG PERIPHERIQUE: QUANTIFICATION DES PROGENITEURS HEMATOPOIETIQUES - MOBILISATION AVANT CYTAPHERESE - BENEFICE SUR LA REPRISE HEMATOPOIETIQUE ET IMMUNITAIRE.

POSITION DU PROBLEME ET BUT DU TRAVAIL :

Le pronostic de nombreuses tumeurs solides agressives est actuellement amélioré par la chimiothérapie intensive associée ou non à la radiothérapie (1,2,3).

Cette escalade des traitements essentiellement myélotoxiques nécéssite l'association d'une greffe de cellules souches hématopoiétiques (CSH), préalablement prélevées chez le patient. L'autogreffe de CSH permet de réduire, sans la supprimer, la période dangereuse de la myélosupression. On peut espérer que l'optimalisation de cette procédure en réduise la mortalité et la morbidité attenantes encore trop élevées et améliore les premiers résultats, déjà prometteurs.

Un projet interuniversitaire, introduit à ce sujet dans le cadre du Télévie 1992 (n° 7.4583.92), est en cours de réalisation. Ce dossier est en fait une demande d'avenant au projet sus-cité.

Les CSH mobilisées et prélevées à partir du sang périphérique présentent certains avantages sur les cellules médullaires :

1. prélèvement moins invasif.
2. possibilité de récolte suffisante de cellules souches même après hémipelvectomie ou irradiation du bassin.
3. reprise hématopoiétique accélérée après greffe (4,5).

Les modalités d'obtention et de prélèvement de ces CSH périphériques doivent cependant encore être précisées, à savoir:

1. le schéma de mobilisation permettant la meilleure récolte de cellules souches d'un point de vue quantitatif et qualitatif : facteurs de croissance combinés entre eux ou non, associés ou non à la chimiothérapie.
2. Le moment idéal de prélèvement de ces cellules par leucaphérèse, établi en fonction des résultats obtenus lors de cycles identiques de mobilisation.
3. la méthode idéale de quantification des CSH (CFU-GM) soit par marquage phénotypique à l'aide d'anticorps monoclonaux (rapide et facile à standardiser), soit par culture (qui apporte des informations fonctionnelles).

En définitive, la meilleure appréciation de la qualité de la procédure de mobilisation et de récolte des cellules souches périphériques se fera par l'étude de la cinétique de reprise hématopoiétique après greffe, en la comparant aux résultats de reprise hématopoiétique après greffe de cellules médullaires. La reconstitution immunitaire, d'intérêt capital également, sera étudiée dans le cadre d'autres projets du laboratoire introduits au F.N.R.S..

RESULTATS PRELIMINAIRES :

Depuis 1992, 25 patients atteints de cancer de mauvais pronostic (lymphome non Hodgkinien, cancer du sein métastasé, tumeur germinale métastasée, cancer bronchique anaplasique à petites cellules) ont été inclus dans un protocole clinique pilote. Ces candidats à une intensification thérapeutique avec autogreffe de CSH et au passé chimiothérapeutique variable, ont reçu, après une cure de chimiothérapie conventionnelle, des facteurs de croissance hématopoiétiques (par voie sous-cutanée) en vue de mobiliser les cellules souches périphériques. Celles-ci ont été récoltées par leucaphérèse (7 litres de sang traités par séance) en fin de traitement aux facteurs de croissance. Certains patients ont bénéficié de 2 cycles de stimulation afin d'augmenter la récolte de CSH périphériques.

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Divers schémas d'administration de facteurs de croissance ont été utilisés :

- Interleukine-3 (7,5 µg/kg/j) associé au GM-CSF (3 ou 5 µg/kg/j) ou au G-CSF (5 ou 12 µg/kg/j).
- GM-CSF seul (5 µg/kg/j)
- G-CSF seul (5 µg/kg/j)

Ces facteurs ont été administrés dans le décours ou à distance de la chimiothérapie. La cinétique de mobilisation des cellules souches hématopoiétiques a été suivie par mise en culture et marquage phénotypique. Les résultats préléminaires sont encourageants :

1. Les facteurs de croissance administrés de façon combinée ou isolée permettent une récolte abondante de cellules souches dans le sang périphérique. Cette récolte est plus importante lorsque les facteurs de croissance sont administrés dans le décours de la chimiothérapie : les taux moyens (+/- SEM) de CFU-GM/kg récoltés par 2 leukaphérèses chez 6 patients traités par une combinaison d'IL-3 et de GM-CSF à distance de la chimiothérapie étaient de 43.426 +/- 20.012 alors que les taux correspondants chez 2 patients traités de la même façon dans le décours immédiat de la chimiothérapie étaient de 523.369 et 4034 CFU-GM/kg (Figure 1).

2. La quantification des CFU-GM par cytofluorométrie en flux après marquage phénotypique (CD34, Ac monoclonal HPCAII de Becton-Dickinson) est corrélée de façon significative à la quantification des colonies dérivées de ces cellules (CFU-GM) en culture (r = 0,69, p = 0,005).

3. Les premiers résultats de la reprise hématopoiétique après greffe sont encourageants : à la fois la reprise de la neutropoièse et de la thrombopoièse semblent accélérée après greffe de cellules souches périphériques associées ou non (1 patient) aux cellules médullaires, comparativement aux résultats de moelle uniquement.

JUSTIFICATION DE LA DEMANDE D'AVENANT

A) Cependant, la mise au point de techniques de quantification des CSH et l'homogénéisation nécéssaire des matériels utilisés dans les trois centres universitaires impliqués dans ce protocole entraîne un surcoût par rapport aux estimations initiales des frais.

- La standardisation des méthodes de culture de CFU-GM selon les méthodes internationales nécéssite l'utilisation de facteurs de croissance (IL-3, GM-CSF, Stem Cell Factor) dont le coût en routine est élevé.

- Par ailleurs, après greffe des CSH périphériques, la reprise plaquettaire semble davantage accélérée que celle des neutrophiles (6). L'explication de cette reprise accélérée nécéssite une quantification précise des précurseurs mégacaryocytaires par mise en culture et marquage par anticorps monoclonal. Ces méthodes de quantification différentes de celles des CFU-GM n'ont pas encore été mises au point : l'optimalisation des milieux de culture par utilisation de facteurs de croissance hématopoiétiques (IL-3, IL-6, IL-11, SCF) et le marquage par anticorps monoclonal des glycoprotéines de membrane (GPIIbIIIa) afin de distinguer ces colonies par cytofluorométrie en flux sont nécéssaires. Ces deux nouvelles méthodes de mesure sont coûteuses en raison de l'utilisation de facteurs de croissance, d'anticorps monoclonal et du matériel de cytofluorométrie en flux qui permet la quantification des colonies.

B) Par ailleurs, le projet comporte un stimulation importante, délibérée de l'hématopoièse à titre thérapeutique. Cette stimulation comporte d'abord le rebond qui suit une aplasie chimio-induite et est ensuite renforcée par l'administration de 2 facteurs de croissance. A court terme ce procédé a déjà démontré son efficacité thérapeutique dans l'autogreffe. Néanmoins, la question est posée quant à l'inocuité du procédé : existe-t-il un risque d'épuisement médullaire à long terme ? Cette question importante mérite

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d'être examinée. Le projet actuel génère une situation particulièrement favorable à son examen et il nous paraît, dès lors, opportun d'ajouter ce volet investigationel au projet. Nous proposons d'étudier, à divers moments de l'étude, (avant chimiothérapie, en aplasie avant facteurs de croissance, après IL-3, après G-CSF, et après retour à l'état de base), les cellules souches pluripotentes dont le maintien doit assurer la perpétuation de l'hématopoièse pendant toute la vie. Nous utiliserons la technique des Pre-CFU ou Delta CFU (7) qui est probablement la seule à même d'approcher, chez l'humain la cellule souche pluripotente capable d'autorenouvellement. La méthode nous permettra d'évaluer le nombre de ces cellules et leur capacité de prolifération.
Ce projet comporte les frais inhérents aux méthodes de culture et à l'usage des facteurs de croissance.

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ROLE DE L'ACTINE ET DES PROTEINES DU CYTOSQUELETTE DANS LA DIFFERENCIATION MEGACARYOCYTAIRE DES AFFECTIONS MALIGNES DE LA MOELLE OSSEUSE

La production des plaquettes sanguines, la mégacaryocytopoïèse, est l'aboutissement d'une prolifération et d'une différentiation de progéniteurs médullaires (les cellules souches) donnant naissance à des précurseurs polyploïdes (les mégacaryocytes) dont le cytoplasme, après maturation, se fragmente en plaquettes [1]. Le contrôle de la mégacaryocytopoïèse est assuré par des facteurs de croissance exerçant une action stimulante ou inhibitrice sur cette hiérarchie de cellules [2].

Au moment où les auteurs pensaient que ce contrôle s'effectuait sur les cellules-souches, nous avons démontré chez l'animal d'expérience que la stimulation de la prolifération des **cellules souches** mégacaryocytaires par le lithium n'est pas due à un effet direct de ces molécules mais bien à la sécrétion de facteurs de croissance par les lymphocytes T [3]. La sécrétion d'acétylcholinestérase par les mégacaryocytes des rongeurs modifie la concentration de l'acétylcholine, un neuromédiateur, et pourrait dès lors jouer un rôle dans la régulation de la production plaquettaire. Cependant, nous avons montré que la stimulation des **cellules-souches** par les agents cholinergiques découle, comme pour le lithium, d'une augmentation de la production de cytokines non spécifiques par les lymphocytes T [4].

Aussi, nos efforts se sont logiquement concentrés sur le phénomène de polyploïdisation mégacaryocytaire. Par notre méthode de mesure du contenu en ADN des mégacaryocytes obtenus en culture de moelle osseuse, nous avons confirmé que l'accroissement de la production plaquettaire suite à l'augmentation des besoins en plaquettes ne s'effectue pas au niveau des **cellules-souches** mais bien à partir des **mégacaryocytes** dont le niveau de ploïdie est proportionnel aux besoins en plaquettes de l'organisme [5]. Notre technique nous a aussi permis de découvrir un progéniteur mégacaryocytaire de faible densité (Light-Density Colony-Forming-Unit Megakaryocyte; LD-CFU-M) dont la probabilité de **polyploïdisation** est plus grande que celle des autres cellules-souches [6].

Dès lors, convaincus de l'importance de la polyploïdisation, nous avons essayé de comprendre ce mécanisme et démontré que l'inhibition de la polymérisation de l'**actine**, une protéine de structure du cytoplasme, entraîne la polyploïdisation mégacaryocytaire qui permet une augmentation de la production plaquettaire [7].

Notre projet a pour but de poursuivre l'étude du mécanisme par lequel la modification de polymérisation de l'actine entraîne la polyploïdisation mégacaryocytaire et d'identifier le désordre de ce mécanisme dans les cancers de la moelle osseuse.

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I. INTRODUCTION.

Les affections malignes de la moelle osseuse se caractérisent par des anomalies de prolifération et de maturation des cellules souches à l'origine des trois lignées des cellules sanguines. La lignée des plaquettes sanguines est issue de cellules de la moelle osseuse, les mégacaryocytes. Ceux-ci tiennent leur nom de la taille importante de leurs noyaux suite à l'acquisition d'un matériel nucléaire polyploïde au cours de la maturation. Dans les leucémies, cancers de la moelle osseuse, les anomalies de la maturation et de la polyploïdisation mégacaryocytaires ainsi que l'action de la chimiothérapie expliquent l'absence de plaquettes fonctionnelles. Comme les plaquettes sanguines sont responsables de l'hémostase primaire, ces patients leucémiques présentent des hémorragies parfois incontrôlables.

La polyploïdisation (endomitose ou endoréduplication) est la règle dans la différenciation normale des mégacaryocytes. Son accentuation assure une augmentation de la production plaquettaire en cas de besoin. En effet, la taille des mégacaryocytes et leur ploïdie augmentent 24 à 48 heures après l'induction d'une thrombopénie chez l'animal. En revanche, l'augmentation provoquée du nombre des plaquettes, par transfusion par exemple, entraîne la diminution de la ploïdie mégacaryocytaire. Il semble que plusieurs substances humorales (IL-6, LIF, IL-11,...) collectivement appelées "thrombopoïétine" exercent une régulation de ce phénomène.

Le mécanisme par lequel s'opère le choix entre la mitose classique et la polyploïdisation est totalement inconnu. Un asynchronisme entre les cinétiques de division nucléaire et cytoplasmique a été invoqué par la plupart des études. Nous avons démontré qu'en conditions contrôlées et stables (cultures de cellules), la cinétique de division nucléaire n'est pas affectée par le type de division mégacaryocytaire (mitose classique ou endomitose). Dès lors, nous avons réfuté l'hypothèse d'une modification de la cinétique de division du noyau dans l'induction du mécanisme de polyploïdisation mégacaryocytaire (1). Les expériences de Paulus, que nous avons confirmées, ont permis d'identifier trois classes de cellules souches mégacaryocytaires en fonction de leurs probabilités (20, 40 et 80%) de quitter la mitose normale pour se polyploïdiser (2,1). Ces travaux ont aussi démontré que les progéniteurs megakaryocytaires diploïdes ont une probabilité stable d'entrer en endomitose durant plusieurs divisions et que celle-ci change au cours d'une seule mitose. Cela suggère que la polyploïdisation mégacaryocytaire résulte de l'altération soudaine d'un mécanisme biochimique impliqué dans la mitose et dont l'action majeure se développe dans le cytoplasme ou la membrane. Grâce à la méthode de mesure de la ploïdie mégacaryocytaire que nous avons développée (3), nous avons isolé et caractérisé un progéniteur mégacaryocytaire de faible densité (LD-CFU-M) dont les probabilités de polyploïdisation sont limitées à deux classes (4). Ces expériences confortent notre hypothèse d'une modification d'un composant cytoplasmique ou membranaire dans l'endomitose.

La séparation cytoplasmique ou cytodiérèse qui termine la mitose normale n'a pas lieu au cours de l'endomitose. La cytodiérèse débute par la contraction d'un anneau sous-membranaire d'actine-myosine. L'actine, la myosine, et la tubuline sont les composants principaux de la structure de soutien et de motilité de la cellule et forment le cytosquelette. Nous avons donc postulé que les protéines du cytosquelette, composants cytoplasmiques et membranaires, sont responsables de la polyploïdisation. Il est vrai que des cellules embryonnaires de nématodes ont été rendues polyploïdes par inhibition de la formation de l'anneau d'actine-myosine (5). Par ailleurs, le fuseau de tubuline détermine le plan de clivage cellulaire et donc l'orientation de l'anneau d'actine-myosine (6,7).

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Récemment, nous avons montré **chez la souris**, que la cytochalasine B, un inhibiteur de la polymérisation de l'actine, ajoutée aux cultures des progéniteurs mégacaryocytaires augmentait la ploïdie moyenne des mégacaryocytes au sein des colonies (8). Par contre, la colchicine, un inhibiteur de la polymérisation de la tubuline n'a pas cet effet. Les techniques de mesure de la ploïdie mégacaryocytaire en culture ne donnent pas de résultats reproductibles **chez l'homme**. Aussi nous avons adopté et modifié une méthode de cytofluorométrie en flux de la ploïdie mégacaryocytaire pour étudier des lignées de cellules cancéreuses à caractère mégacaryocytaire. L'addition d'esters de phorbol (stimulants non spécifiques) et/ou de cytochalasine B (inhibiteur de la polymérisation de l'actine) aux milieux de culture de certaines lignées humaines (DAMI, HEL, K 562 et MEG 01), entraînent une augmentation significative de l'endomitose mégacaryocytaire. Il semble donc que l'absence de polymérisation de l'actine soit nécessaire à l'augmentation de ploïdie des mégacaryocytes. Ceci pourrait être expliqué par l'absence de formation en télophase de l'anneau contractile d'actine-myosine qui mène habituellement à la séparation cytoplasmique. Comme la concentration de l'actine au sein de la cellule est constante, il est évident qu'au cours de la division cellulaire, la synthèse d'actine nouvelle au sein d'une cellule est un phénomène contrôlé.

Comment envisager le rôle de l'actine dans le phénomène de polyploïdisation? Nous savons que les esters de phorbols (en particulier, le phorbol myristate acétate ou PMA) agissent sur la cellule en stimulant des récepteurs de manière non spécifique. L'activation de ces récepteurs dont le cytosquelette est partie intégrante peut amener diverses réactions inhibitrices ou stimulantes au sein de la cellule. Comme la réponse de la cellule suite à l'action du PMA n'est pas aussi importante que celle produite par la cytochalasine B, nous pensons que l'augmentation de ploïdie par la cytochalasine B doit être expliquée par l'action maximale sur un mécanisme effecteur terminal partiellement modulable par les récepteurs aux diverses cytokines.

Par quel mécanisme, le besoin accru en plaquettes et dès lors l'engagement dans le phénomène de polyploïdisation mégacaryocytaire peuvent mener à la diminution de polymérisation de l'actine? Tout d'abord, l'expression génique de l'actine, si elle est contrôlée devrait logiquement dépendre du produit final sous l'une ou l'autre de ses formes (actine G et/ou actine F). Toute modification d'un des composants (actine F) pourrait modifier la synthèse finale d'actine. Ensuite, la thymosine B4 a la propriété de séquestrer la majorité de l'actine G soluble (9). Elle peut donc moduler l'équilibre entre formes soluble et polymérisée de l'actine qui serait responsable du maintien d'une division mitotique normale. Enfin, le type particulier de polymérisation de l'actine pourrait aussi intervenir dans ce phénomène. En effet, la polymérisation peut être schématisée comme une lente progression d'une extrémité (arrow) du filament d'actine F où les monomères d'actine viennent se lier par rapport à l'autre extrémité (tail) dont les monomères d'actine se détachent. Donc, avec un équilibre F/G conservé, le type chimique d'actine peut encore être chimiquement modifié par ce processus dynamique au sein de la molécule déjà polymérisée.

II. BUT DU TRAVAIL.

Notre projet a pour but d'une part de préciser le mécanisme par lequel la modification de polymérisation de l'actine dans le cytoplasme engendre l'endomitose mégacaryocytaire et d'autre part de déceler, dans certains cancers de la moelle osseuse où la mégacaryocytopoïèse est déficiente, les anomalies du métabolisme des protéines du cytosquelette.

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III. APPROCHE EXPERIMENTALE.

A. ETABLISSEMENT DE LIGNEES MEGACARYOCYTAIRES HUMAINES.

Nous avons pu obtenir des lignées continues de cellules mégacaryocytaires leucémiques (DAMI, MEG01, HEL, K562). Notre laboratoire a développé quatre nouvelles lignées à partir d'affections malignes de la moelle osseuse (2 de Leucémie Myéloïde Chronique et deux de Thrombocytémie Essentielle). Ces lignées doivent encore être caractérisées. En particulier, leurs quantités mégacaryocytaires (expression des glycoprotéines IIb/IIIa et Ib) à l'état basal et après stimulation par PMA seront étudiées par cytofluorométrie en flux.

B. ETUDE DE LA CONCENTRATION D'ACTINE INTRACELLULAIRE.

1. MESURE GLOBALE AU CYTOFLUOROMETRE EN FLUX.

La concentration intracellulaire d'actine sera estimée par cytofluorométrie en flux (FACScan). Nous avons pu établir la mesure de l'actine intracytoplasmique de populations lymphocytaires d'anticorps monoclonaux couplés à la fluorescéine. Ce fluorochrome émettant une lumière verte après excitation dans l'ultraviolet permet la mesure concomitante de la ploïdie par l'iodure de propidium qui, avec la même fréquence d'excitation émet une lumière rouge. Nous étudierons la concentration d'actine dans les cellules des lignées cancéreuses à composante mégacaryocytaire. Nous corrèlerons cette concentration à la ploïdie de chaque cellule. Trois possibilités peuvent être envisagées. Tout d'abord, la découverte d'une absence d'augmentation de la concentration d'actine au cours de l'endomitose suggérerait que, globalement, la plus grande partie de l'actine est impliquée dans le phénomène d'endomitose. Par contre, une augmentation progressive ou une augmentation "saltatoire" suggèreraient qu'une faible partie seulement de l'actine serait impliquée dans l'endoréduplication.

La distribution de l'actine des mégacaryocytes provenant de la moelle osseuse de donneurs de greffe permettra de tester notre hypothèse au cours de la régulation physiologique de cellules mégacaryocytaires normales.

2. MESURE QUANTITATIVE DE L'ACTINE G ET F PAR "CELL SORTER".

L'actine se présente sous deux formes : monomère soluble (actine G) ou polymère insoluble (actine F). Les monomères d'actine sont très hétérogènes et se caractérisent surtout par les sites de liaison aux ligands (par exemple la gelsoline et la profiline pour la structure; la myosine pour la motilité et l'ATP pour apporter l'énergie contractile). Malgré cette hétérogénéité qui définit les fonctions de l'actine polymérisée, la concentration d'actine est relativement constante dans toutes les cellules. C'est d'ailleurs pour cette raison qu'elle a été utilisée comme contrôle de l'expression génique d'autres molécules. Quelques anticorps monoclonaux reconnaissent différents épitopes de l'actine. Malheureusement, ils ne permettent pas de détecter réellement les sites de réaction des ligands et dès lors ne permettent pas de mesurer les différents types d'actine. Aussi, en marquant par exemple en fluorescence les ligands eux-mêmes, nous pourrons déterminer dans l'endomitose, le type chimique d'actine en cause et, par conséquent la fonction perturbée (structure, motilité ou énergie). Par ailleurs, comme nous l'avons expliqué, la forme F est en constant renouvellement par la synthèse en "arrow" et la destruction en "tail". Le contrôle de ce phénomène dynamique est encore mal connu mais peut être étudié grâce à la phalloïdine dont la propriété est de se fixer spécifiquement à une extrémité de la molécule polymérisée et de bloquer définitivement la polymérisation. La phalloïdine peut facilement être couplée à une molécule fluorescente.

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Dès lors, pour mettre en évidence le type précis d'actine impliqué dans la polyploïdisation, nous emploierons le tri cellulaire au cytofluoromètre (COULTER ELITE) des cellules des lignées ainsi que des cellules normales. Nous avons déjà réalisé la séparation de mégacaryocytes en diverses phases endomitotiques (2N versus >2N). Nous pourrons ainsi déterminer par électrophorèse des extraits de cellules triées en classes de ploïdie, les modifications qualitatives et quantitatives de l'actine. Ceci sera aussi réalisé sur les lignées mégacaryocytaires après stimulation par les esters du phorbol et par des cytokines telles que l'interleukine-6.

C. DETERMINATION DES ANOMALIES DE LA MATURATION MEGACARYOCYTAIRE DANS LES AFFECTIONS MALIGNES DE LA MOELLE OSSEUSE.

Par les techniques mises au point sur les lignées cellulaires et décrites plus haut, nous étudierons au fur et à mesure des pathologies rencontrées en clinique, le rôle de l'actine dans la régulation anormale des leucémies et états apparentés. Nous focaliserons nos efforts sur l'étude de la polyploïdisation classiquement décrite dans les syndromes myéloprolifératifs tels que la polyglobulie de Vaquez et la thrombocytémie essentielle et dans les pathologies cancéreuses de l'hématopoïèse (Leucémies aiguës à caractère mégacaryocytaire).

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DETECTION PAR AMPLIFICATION GENIQUE DES MICROMETASTASES MEDULLAIRES ET DE CONTAMINATION DE CELLULES SOUCHES DU SANG PERIPHERIQUE PAR DES CELLULES DE TUMEURS SOLIDES.

Notre laboratoire s'intéresse depuis de nombreuses années à l'autogreffe de moelle osseuse après chimiothérapie intensive dans le traitement des tumeurs solides. Il était logique de chercher à déceler un envahissement médullaire pour prévenir une contamination possible d'un prélèvement de moelle utilisé en vue d'une autogreffe. Les cellules de cancer bronchique anaplasique à petites cellules ont été détectées au sein de la moelle par des anticorps monoclonaux et par la technique de culture clonogénique de cellules tumorales (1,2,3). Ces contaminations médullaires posent le problème de l'utilité de la "purge du greffon" (4,5).

Actuellement, les cellules souches hématopoïétiques du sang périphérique tendent à remplacer les cellules médullaires dans la greffe autologue surtout lorsque la moelle est contaminée par des cellules néoplasiques ou lorsque celle-ci est fibrosée ou appauvrie suite aux chimio- et radiothérapies. Beaucoup de nos patients bénéficient actuellement de ce type de greffe. L'utilisation de facteurs de croissance hématopoïétiques dont nous avons acquis l'expérience clinique (6) a facilité la transplantation de cellules souches circulantes. Enfin, notre laboratoire possède une expertise dans l'utilisation de la technique d'amplification génique (7). Nous proposons d'utiliser cette technique afin de détecter des cellules tumorales dans la moelle et le sang de patients atteints de cancer bronchique anaplasique et de cancer du sein.

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INTRODUCTION

1. Détection de métastases occultes dans la moelle osseuse

La détection de métastases médullaires de tumeurs solides présente un intérêt pour le pronostic de la maladie néoplasique au moment du diagnostic et pour le suivi des patients traités. Diverses études ont montré la fréquence non négligeable de métastases osseuses du cancer du sein lors du diagnostic. Si plus de 40% des patientes avec un cancer du sein métastatique ont un envahissement de la moelle osseuse détecté par des méthodes habituelles (ponction-biopsie médullaire) (1,2), 28% des patientes sans métastase osseuse documentée par des critères habituels (scintigraphie et radiographie osseuses) ont un envahissement médullaire déterminé par des techniques d'immunocytochimie non spécifique (3). In vitro, on a pu révéler en culture des cellules tumorales dans les moelles osseuses histologiquement normales de patientes atteintes d'un cancer du sein (4). Des cellules tumorales peuvent être détectées dans la moelle osseuse par immunofluorescence chez 17% des patientes ayant un cancer du sein sans envahissement ganglionnaire à l'histologie (5). Plus récemment, 260 patientes atteints d'un cancer mammaire non métastatique ont subi une ponction médullaire au moment de l'exérèse de la tumeur primitive. Par une technique d'immunofluorescence, des cellules tumorales ont été identifiées dans la moelle osseuse de 44% de ces patientes (6). La détection des cellules néoplasiques apparaissait, dans cette étude, constituer un facteur de prédiction de rechute à distance de la maladie et donc un facteur de pronostic.

Le cancer bronchique à petites cellules présente des caractéristiques cliniques morphologiques et biologiques particulières. Sa propension à la dissémination précoce à distance est bien connue. La présence de cellules tumorales au niveau de la moelle osseuse peut constituer un facteur de pronostic. Notre laboratoire a montré par l'utilisation des techniques de culture clonogénique de cellules tumorales et d'immunofluorescence indirecte, l'existence d'infiltration médullaire par des cellules de carcinome anaplasique bronchique échappant à l'analyse morphologique classique (7,8,9).

2. Contamination des cellules souches périphériques

Les cellules souches du sang périphérique sont de plus en plus utilisées dans le traitement de tumeurs solides disséminées. Les premières réinfusions des cellules souches du sang circulant ont été administrées pour la reconstitution hématopoiétique potentielle chez les patients cancéreux qui ne pouvaient bénéficier d'une autogreffe de moelle osseuse en raison d'une maladie résiduelle, ou parce que la moelle était trop fibrosée pour permettre la collection d'un nombre adéquat de cellules. Le risque de contamination par des cellules tumorales est généralement considéré comme minime. Cependant, la circulation de cellules tumorales a été documentée pour le neuroblastome et le cancer du sein (10,11,12). Dans le cas du neuroblastome, la concentration de cellules néoplasiques dans le sang lors du diagnostic peut être élevée : de 2 à $1000/10^5$ cellules mononuclées. Les cellules souches du sang périphérique utilisées en vue d'autogreffe chez des patients atteints de neuroblastome sont fréquemment contaminées : parmi 8 patients étudiés, 2 présentaient des cellules tumorales au sein des cellules souches. Chez des patientes atteintes de cancer du sein, et candidates à une autogreffe, des cellules néoplasiques ont été recherchées dans les prélèvements de cellules souches périphériques. Par analyse immunocytologique, des cellules de cancer du sein sont décelées dans 4/37 leucaphérèses chez 20 patientes (11).

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3. Méthodes de détection

Les techniques utilisées le plus communément afin de détecter des cellules tumorales au sein des tissus hématopoïétiques font appel à toute une panoplie d'anticorps monoclonaux qui souvent ne sont pas spécifiques d'une tumeur. Cette immunodétection est assez sensible (1 cellule sur 100.000) mais peut-être subjective. Nous utiliserons la méthode d'amplification génique en chaîne (PCR) afin de déceler des contaminations possibles de la moelle osseuse ou des cellules souches périphériques par des cellules tumorales.

Dans le cas des tumeurs solides, il existe quelques rares anomalies cytogénétiques récurrentes telles que la délétion d'une partie du chromosome 3 dans le cancer bronchique anaplasique et la translocation 11;22 dans le sarcome de Ewing. En ce qui concerne les deux types de tumeurs (le cancer du sein et le cancer bronchique anaplasique) qui seront investigués, les anomalies cytogénétiques ne sont pas exploitables pour la technique de PCR. Nous rechercherons donc des gènes exprimés exclusivement par les cellules de ces tumeurs.

4. Signification de la détection de cellules tumorales

Si les techniques de PCR se sont avérées impressionnantes dans la détection de la maladie résiduelle, notamment dans la détection de translocations présentes dans certaines affections néoplasiques hématologiques, leur utilité clinique reste à démontrer.

Il faut considérer d'une part l'aspect pronostique de cette détection au moment du diagnostic d'une tumeur solide et d'autre part la signification de la détection dans les cellules souches périphériques.

En effet, ces problèmes de contamination possible des cellules souches périphériques peuvent soulever d'autres questions :

1. Faut-il détecter systématiquement des cellules néoplasiques dans les cellules souches ?

2. Quelle méthode fiable faut-il utiliser ?

3. Quelle est la signification d'une contamination éventuelle ? Faut-il modifier le traitement?

4. Faut-il purger les cellules souches des cellules tumorales (13,14) ou faut-il enrichir les cellules souches en concentrant des cellules hématopoïétiques (CD34 p.ex.) pures qui seraient moins contaminées ?

BUT DU TRAVAIL

a) Mettre au point une méthode sensible et fiable de détection par PCR de cellules de cancer mammaire et de cancer anaplasique pulmonaire parmi des cellules hématopoïétiques; cette technique devrait être plus performante que l'utilisation d'anticorps monoclonaux.

b) Utiliser cette méthode afin de détecter dans la moelle osseuse des micrométastases de ce type de tumeurs lors du diagnostic ou à la rechute de la maladie.

c) Détecter la contamination possible des cellules souches hématopoiétiques circulantes par des cellules tumorales.

d) Définir la corrélation entre la détection de cellules malignes dans la moelle ou le sang et l'évolution de la maladie (en d'autre termes la rechute de la maladie néoplasique est-elle plus précoce chez les patientes présentant des métastases occultes ; faut-il traiter plus agressivement les patients porteurs de micrométastases?).

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APPROCHE EXPERIMENTALE

a) La première étape consiste à déterminer quels sont les gènes exprimés fréquemment et exclusivement par les cellules cancéreuses et non par le tissu hématopoïétique. En effet, à la différence des tumeurs hématologiques telles que les leucémies et les lymphomes, il n'y a pas de translocations récurrentes qui puissent être exploitées. L'expression de différents gènes dont la séquence est connue sera analysée dans des lignées tumorales de cancer du sein et du poumon. Ils seront choisis a priori en considérant que leur expression doit être spécifique d'une tumeur solide et non décelable dans un tissu hématopoiétique. Pour les cancers du sein par exemple, on peut retenir les gènes encodant la mucine exprimée par plus de 90% des cancers du sein, l'oncogène c-Erb b2/Neu, les gènes encodant les récepteurs aux oestrogènes et progestérone. Pour le cancer du poumon, un antigène exprimé par le tissu cérébral et les cellules de cancer anaplasique pulmonaire a été cloné (15) et pourrait être utilisé dans la détection de ces cellules néoplasiques.

b) Une série de lignées tumorales sera étudiée afin de sélectionner les gènes exprimés fréquemment par ces lignées. Le cDNA synthétisé à partir d'ARN est amplifié par PCR. Les cellules tumorales de lignées seront ensuite diluées dans du tissu hématopoïétique sain afin de déterminer la limite de détection des cellules tumorales (1/100-1/1.000.000). Afin d'augmenter la sensibilité de la PCR, les amorces utilisées seront doubles : un aliquot du premier produit de PCR sera utilisé pour une deuxième PCR en utilisant des amorces situées "à l'intérieur" des amorces de la première PCR. Donc, cette seconde PCR sera enrichie en séquence recherchée suite à la PCR initiale. Les amorces de la deuxième PCR ("nested primers") peuvent éliminer le background et permettent d'obtenir un meilleur produit final. Des contrôles doivent être régulièrement utilisés car la technique peut être grevée d'une fréquence non négligeable de faux positifs. Par ailleurs, il faut s'assurer que des cellules médullaires non hématologiques telles que les fibroblastes ou les cellules endothéliales ne produisent pas les gènes utilisés pour la détection de cellules tumorales. Les avantages de la PCR sont la rapidité de la procédure et la possibilité de détecter des cellules malignes à une haute dilution (dans le cas de leucémies par exemple, 1 cellule sur 10^6 serait détectable (16)).

c) L'évolution clinique des patients présentant ou ne présentant pas de métastases médullaires sera suivie. Nous pouvons ainsi préciser l'importance de cette détection dans le pronostic et la thérapeutique de la maladie.

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ETUDE DE LA RESTAURATION IMMUNITAIRE APRES TRANSPLANTATION DE CELLULES SOUCHES HEMATOPOIETIQUES CHEZ LA SOURIS

Description succinte du projet de recherches (2 ou 3 pages maximum)

L'exposé du projet de recherches que l'on envisage de réaliser doit être précédé d'une brève description de l'hypothèse de travail.

Dans l'éventualité où ce projet devrait s'étendre sur plusieurs exercices, il y a lieu d'en indiquer clairement les phases successives avec leur répartitions dans le temps ainsi que les corrélations entre l'objectif à atteindre et les crédits sollicités.

Indiquez le nombre **d'années académiques** pour lesquelles ce projet est sollicité : 2 ans

INTRODUCTION

La chimiothérapie intensive suivie d'autogreffe de moelle osseuse ou de cellules souches hématopoiétiques du sang périphérique permet d'obtenir des rémissions complètes. Toutefois, il existe un taux élevé de rechutes particulièrement précoces. L'éradication de la maladie résiduelle est d'autant plus difficile que la période post-greffe s'accompagne d'une immunodépression. Nous avons pu investiguer certains signaux de transduction de l'activation précoce des lymphocytes T CD4+ ainsi que différentes voies de stimulation de ces lymphocytes chez des patients autogreffés (1,2). Ces travaux commencent à nous révéler une partie des mécanismes impliqués dans le déficit immunitaire post-autogreffe. Nous sommes souvent confrontés dans l'analyse de l'investigation in vitro de l'immunité post-greffe de ces patients à une variabilité des résultats due vraisemblablement à l'hétérogénéité de la population étudiée. Ces patients ont des tumeurs différentes et par conséquent ont reçu des chimiothérapies différentes avec leurs effets myélosuppresseurs propres. Nous proposons d'utiliser un modèle animal de transplantation de moelle osseuse entre souris syngéniques afin de pouvoir disséquer de manière plus systématique l'immunité post-greffe. Au cours des dernières années, la connaissance des mécanismes d'activation précoce des lymphocytes T s'est étendue.

Le déficit immunitaire après autogreffe de moelle osseuse atteint principalement les lymphocytes T. Jusqu'il y a quelques années, on pouvait résumer ce déficit cellulaire en quelques observations classiques : une réduction des taux relatifs et absolus de lymphocytes T CD4+ dans le sang périphérique associée à un taux normal voire augmenté du nombre de lymphocytes CD8+, un défaut de prolifération des lymphocytes T et une diminution de la sécrétion d'interleukine-2. Nous avons étudié par cytofluorométrie en flux l'augmentation de calcium intracellulaire au niveau des lymphocytes CD4+ après stimulation par la concanavaline A par l'utilisation concomitante d'un colorant spécifique du calcium, le fluo-3 et un anticorps monoclonal reconnaissant la sous-population CD4+ (1). Physiologiquement, l'activation des lymphocytes T entraîne suite à l'hydrolyse du phosphatidylinositol 4,5-biphosphate par la phospholipase C, la formation de 2 produits : l'inositol 1,4,5-triphosphate qui va provoquer une augmentation de calcium intracellulaire et le diacyl-glycerol qui active la protéine kinase C. Chez les patients autogreffés depuis moins d'un an, on observe une augmentation plus faible du taux de calcium intracellulaire au niveau des lymphocytes T CD4+ par rapport à celle observée chez des sujets contrôles. La diminution semble s'expliquer par une réduction du nombre de cellules capables d'augmenter leur taux de calcium intracellulaire normalement. Chez les patients greffés depuis plus d'un an, le taux de calcium intracellulaire moyen est plus faible dans les lymphocytes T activés par rapport à un groupe de sujets sains mais cette différence n'est pas statistiquement significative. Ceci suggère qu'il existe une anomalie dans la formation des signaux de transduction précoce après stimulation et que ce phénomène tend à régresser à distance de la greffe. Nous avons également étudié la production d'IL-2 après stimulation par PHA, PHA + PMA, PMA + un ionophore du calcium, et PMA + anti CD28 (2). Ces différents stimuli induisent une production d'interleukine-2 inférieure à celle observée dans un groupe d'individus normaux. La production d'ARNm d'IL-2 apparaît aussi réduite. Ceci indique que différentes voies d'activation des lymphocytes T ne fonctionnent pas normalement après greffe de moelle osseuse. Par contre, le récepteur de l'IL-2 est normalement exprimé par les lymphocytes T après activation (3,4) et ceux-ci répondent à l'adjonction d'IL-2 in vitro ce qui suggère que cette voie d'activation reste

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préservée. La réduction de la production d'IL-2 en présence de PMA et d'anti-CD28 correspond à l'observation récente d'une réduction de l'expression de l'antigène CD28 par les lymphocytes TCD3+ des patients autogreffés (5) et la diminution de prolifération en présence d'anti-CD28. La quantité de cytokine produite par les lymphocytes CD4+ durant une stimulation antigénique est contrôlée par des signaux co-stimulants dépendant des cellules présentant l'antigène. L'expression de la molécule B7, le ligand du CD28, apparaît critique dans la capacité de co-stimuler les lymphocytes T.

Le déficit de prolifération des lymphocytes T post-greffe ressemble au phénomène d'anergie clonale, modèle faisant appel au concept de co-stimulation par deux signaux nécessaires à l'activation de lymphocytes T. Ce modèle a été développé notamment pour expliquer la non-réponse immunitaire à un auto-antigène. Il existe de plus en plus d'arguments pour penser que l'interaction B7-CD28 constitue le deuxième signal co-stimulant des lymphocytes T. Dans le cas de l'autogreffe, la présence de monocytes allogéniques obtenus chez des sujets sains ne semble pas améliorer la prolifération et la sécrétion d'IL-2 (6). Nous pensons que la diminution du CD28 à la surface des lymphocytes T et/ou l'anomalie de cette voie d'activation peut induire un phénomène semblable d'anergie au niveau des cellules T.

L'activation des lymphocytes T menant à la production de cytokines est un phénomène dont les mécanismes sont peu à peu élucidés. Le récepteur de l'antigène est constitué par une association hautement complexe d'oligomères comprenant au moins 7 protéines membranaires distinctes. Le récepteur T doit d'abord reconnaître et se lier à son ligand. Cette liaison se traduit ensuite en signaux intracellulaires résultant finalement en une réponse appropriée. La spécificité à l'antigène est définie par deux chaînes variables α et β. D'autres molécules non variables dont les fonctions restent imprécises sont associées aux récepteurs T : CD3 (gamma, δ, ϵ), ζ et η. Ces différentes chaînes semblent faire le lien entre la reconnaissance de l'antigène et les transferts des signaux de transduction intracellulaires (7). Le dimère ζ apparaît important dans la transduction des signaux. Son domaine cytoplasmique a la capacité de transmettre les signaux indépendamment des autres chaînes du récepteur T. La stimulation du récepteur T active la protéine tyrosine kinase entraînant la phosphorylation de multiples protéines cellulaires. L'inhibition de ce phénomène provoque une diminution de la production d'IL-2. Deux protéine tyrosine kinases, particulières, fyn et lck, sont impliquées dans la fonction du récepteur T et jouent un rôle dans la transmission de l'information.

Notre projet est focalisé sur ces aspects d'activation des lymphocytes T, afin de distinguer les molécules défectueuses dans la chaîne de transmission des signaux et peut-être de pouvoir y remédier et d'ainsi corriger l'immunité post-greffe.

BUT DU PROJET

Etudier l'activation des lymphocytes T après greffe de moelle syngénique chez les souris sous différents aspects:

1. Mesurer la présence des molécules associées au complexe récepteur T et leur rôle dans le déficit immunitaire post-greffe.

2. Analyser la voie d'activation lymphocytaire T par le CD28 après greffe de moelle.

3. Analyser l'expression des tyrosine kinases p56 lck et p59 fyn par les populations CD4+ et CD8+

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APPROCHE EXPERIMENTALE

La transplantation de moelle entre souris syngéniques se fera selon des protocoles tout à fait classique. Après irradiation, les souris recevront la moelle par voie intraveineuse. Les souris receveuses seront sacrifiées tous les 15 jours afin d'étudier :

1. la répartition des sous-populations de lymphocytes T au niveau du sang périphérique et la prolifération par incorporation de [^{3}H]-thymidine suite à la stimulation par différents mitogène dont l'anti-CD28.

2. La présence des protéines associées au complexe récepteur T au niveau des lymphocytes CD4+ post-greffe. Ceux-ci sont isolés afin d'obtenir une population pure. Ces cellules sont marquées radioactivement puis lysées. Le complexe récepteur T est précipité à partir du surnageant des cellules lysées avec un anticorps anti-CD3 et séparé sur gel de polyacrylamide à 2 dimensions. La protéine ζ peut également être visualisée par immunoblot. Les protéines du surnageant des cellules lysées sont séparées par électrophorèse transférées sur membrane, puis détectées avec anticorps anti-ζ.

3. Les protéines lck et fyn. Celles-ci seront également analysées par immunoblot, à partir des lymphocytes CD4 isolés. Par ailleurs, l'activité kinase de ces protéines sera étudiée. Ces protéine kinases peuvent être obtenues à partir des lysats cellulaires en utilisant un anticorps polyclonal formé contre ces protéines. Les complexes immuns obtenus sont récoltés et placés en présence d'ATP marqué pour la réaction de kinase. Les polypeptides phosphorylés sont ensuite séparés sur gel. L'activité kinase peut être quantifiée par densitométrie en comparaison avec celle des cellules de sujets normaux.

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UTILISATION THERAPEUTIQUE DE TRANSFERT DE GENE PAR VECTEUR RETROVIRAL DANS DES CELLULES SOUCHES HEMATOPOIETIQUES CIRCULANTES: ETABLISSEMENT D'UN MODELE PRE-CLINIQUE MURIN ET DEFINITION DES CONDITIONS IN VITRO DE TRANSFECTION PAR RETROVIRUS DES CELLULES SOUCHES HUMAINES.

Nous avons publié la première étude clinique prospective et randomisée concernant l'intérêt de l'intensification thérapeutique sous couvert d'autogreffe de moelle osseuse dans le cancer anaplasique à petites cellules (1) et défini les conditions de concentration des cellules souches hématopoïétiques médullaires (2). Après autogreffe, il existe un déficit immunitaire pouvant persister plus d'un an. Nous avons démontré que la capacité de prolifération des lymphocytes T réduite après greffe peut être restaurée in vitro par l'Interleukine-2 (IL-2) (3). Nous avons également observé que la production d'IL-2 par les lymphocytes T est diminuée de même que la production de l'ARNm de l'IL-2 par rapport aux contrôles normaux (4). De même l'élévation du calcium intra-cellulaire suivant stimulation par concanavalin A est réduite (5). Nous avons ensuite montré que l'administration systémique d'IL-2 ne corrige que partiellement ces déficits chez les greffés (6).

Les cellules souches hématopoïétiques périphériques permettent une reprise après greffe plus rapide que les cellules médullaires. Notre équipe cherche à optimaliser les schémas d'administration de facteurs de croissance après chimiothérapie permettant d'obtenir les meilleures collectes de cellules souches périphériques par leucaphérèse (7). Les caractéristiques biologiques des cellules souches et leur abondance en font des cibles idéales pour un transfert de gène par vecteurs rétroviraux. Dans un modèle murin préclinique, nous tenterons de corriger le déficit immunitaire après greffe en introduisant, grâce à un rétrovirus, le gène de l'IL-2 ou du CD4 dans des cellules souches périphériques murines. Nous définirons également *in vitro* les conditions optimales de transfection par rétrovirus de cellules souches périphériques humaines collectées en vue d'une greffe.

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L'existence d'une relation dose-réponse en chimiothérapie anticancéreuse est le point de départ des essais cliniques d'escalade de dose, encore appelés "intensification thérapeutique". La toxicité limitante de la plupart des agents cytostatiques étant la myélotoxicité, les intensifications thérapeutiques se pratiquent sous le couvert d'auto- ou d'allo-greffe de cellules souches hématopoïétiques afin de prévenir une aplasie médullaire irréversible. Notre équipe est la première à avoir publié une étude clinique prospective et randomisée concernant l'intérêt de l'intensification thérapeutique dans le cancer bronchique anaplasique à petites cellules (1) et aujourd'hui les chimiothérapies intensives avec autogreffe de cellules souches hématopoïétiques sont activement investiguées dans le traitement des leucémies, des lymphomes, des cancers du sein, du testicule ainsi que de l'ovaire (2).

La chimiothérapie intensive suivie de greffe de cellules souches hématopoïétiques qui ne comprennent qu'un nombre limité de précurseurs lymphoïdes a pour conséquence d'induire chez les malades une immunosuppression prolongée, de plus d'un an, contribuant vraisemblablement aux infections opportunistes et aux rechutes tumorales fréquemment rencontrées chez les malades ainsi traités. Notre équipe a contribué à la caractérisation de ce déficit immunitaire (diminution du pourcentage et du nombre absolu de cellules T CD4+, augmentation du nombre absolu et relatif des cellules NK CD56+, déficit lymphoprolifératif T, déficit de production d'IL-2) et au développement de ses moyens de correction (3,4). L'utilisation systémique d'IL-2 comme immunothérapie adjuvante après intensification thérapeutique et greffe de cellules souches hématopoïétiques produit des effets biologiques intéressants sur les cellules NK CD56+ mais peu marqués en ce qui concerne les lymphocytes T (5). En raison de la toxicité de l'IL-2, ce traitement n'est pas sans risque et son application au long cours est difficilement pratiquable.

On trouve chez l'homme comme chez la souris des cellules souches capables de repeupler le système hématopoïétique après agression léthale, dans le foie de foetus, dans le sang de cordon ombilical, dans la moelle osseuse et dans le sang périphérique de l'adulte (6). L'utilisation de cellules souches hématopoïétiques du sang périphérique est aujourd'hui activement investiguée (projet interuniversitaire FRSM 74.583.92) en raison de leurs propriétés intéressantes en ce qui concerne la cinétique de régénération hématopoïétique après greffe ou en raison de leur collecte facile par leucaphérèses pratiquées en période de régénération hématopoïétique post-chimiothérapique après administration de facteurs de croissance hématopoïétiques. Les cellules souches ainsi collectées présentent une propriété biologique intéressante supplémentaire : une haute proportion d'entre elles est activement engagée dans le cycle de prolifération cellulaire ce qui en fait des cibles idéales pour transfection par vecteur rétroviral (7) contenant par exemple le gène de l'IL-2 ou le gène du CD4.

Le choix des cellules souches périphériques pour tentative de correction du déficit immunitaire post-greffe par transfert de gène apparaît encore plus judicieux si l'on considère que les leucaphérèses pratiquées après chimiothérapie et stimulation hématopoïétique collectent une fraction déjà concentrée *in vivo* (jusqu'à 20% des cellules mononucléées ainsi collectées sont CD34+) (8). De plus ces progéniteurs hématopoïétiques sont relativement plus mûrs que ceux de la moelle osseuse, ce qui explique qu'une bonne proportion d'entre eux est appelée à s'éteindre dans les mois qui suivent la greffe. Il s'agit là d'une propriété intéressante à exploiter si l'on souhaite que l'expression du gène greffé s'amenuise avec le temps après la greffe. En revanche, la question de la reprise hématopoïétique à long terme dans les cas de greffes pratiquées avec les seules cellules souches hématopoïétiques périphériques n'est pas encore résolue (9) et la contribution de ce type de greffon à l'hématopoïèse post-greffe devrait trouver une réponse au travers du type de manipulation que nous envisageons.

Dans une étude clinique réalisée par notre groupe (5), l'administration d'IL-2 augmente l'activité des cellules NK CD56+ jusqu'à 2 semaines après arrêt de la thérapeutique mais ne restaure pas de manière significative les capacités de prolifération des lymphocytes T, contrairement à ce que nous avions observé *in vitro* (3). On peut espérer que la transfection d'un gène d'IL-2 ou de CD4 dans des cellules souches périphériques greffées entraînera un effet biologique et clinique plus marqué car le récepteur de l'IL-2 est normalement exprimé à la surface des lymphocytes T qui réapparaissent dans le sang après transplantation de cellules souches. Par ailleurs, l'importance de la majoration de l'activité cytotoxique non MHC-restreinte dans un contexte de maladie résiduelle n'est pas clairement établie; toutefois pour plusieurs types de

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tumeurs, l'utilisation d'IL-2 associée ou non à l'administration de lymphocyte activated killer (LAK) ou tumor infiltrating lymphocyte (TIL) s'est révélée capable d'induire des regressions tumorales (10). Il apparaît que l'IL-2 joue un rôle central dans le déficit immunitaire après autogreffe et dans l'immunothérapie adoptive anticancéreuse, ce qui justifie pleinement l'emploi de cette cytokine dans notre modèle.

Les lymphocytes T helper, principaux producteurs d'IL-2, sont classiquement caractérisés par leur phénotype CD4. La population CD4+ est nettement réduite chez les patients autogreffés et cette diminution du nombre absolu de cellules CD4+ explique en partie le déficit de secrétion d'IL-2. Le rôle de la molécule d'adhésion CD4 n'est pas entièrement élucidé. Son intégrité apparaît nécessaire pour une réponse immunitaire normale, car elle interagit avec certaines tyrosine kinases liées au complexe récepteur T. Elle permettrait d'amplifier la production d'IL-2. De plus, la présence à faible densité de l'antigène CD4 décrite à la surface des cellules pluripotentes hématopoiétiques suggère que cette molécule joue également un rôle dans l'hématopoïèse au sens large. Les effets de la transfection du gène encodant la molécule CD4 dans les cellules souches hématopoiétiques seront également très intéressants à investiguer.

BUT DU PROJET

L'objectif de ce travail est d'une part de développer un modèle pré-clinique de transfection de cellules souches hématopoïétiques murines avec des vecteurs rétroviraux contenant le gène de l'IL-2 ou du CD4 afin de tester les aspects de faisabilité, de toxicité et le retentissement de ces manipulations sur la reconstitution immunitaire et la régénération hématopoïétique au long cours après greffe. Les vecteurs seront, si nécessaire, rendus plus contrôlables par l'adjonction d'un gène-suicide comme, par exemple, le gène de la cytosine déaminase qui convertit la 5-fluorocytosine non toxique en 5-fluorouracil cytotoxique (11). Ceci permettrait d'éliminer les cellules transfectées en cas d'accident non anticipé.

D'autre part, le projet a pour but la production de vecteurs rétroviraux totalement dépourvus de particules virales recombinantes et de tester la possibilité de transfecter, *in vitro*, des cellules souches périphériques humaines collectées dans des conditions similaires à celles d'un protocole clinique.

Une éventuelle application clinique ferait bien sûr l'objet d'une demande d'avenant au présent projet et ne serait envisagée qu'après obtention préalable des autorisations requises pour une telle expérience.

APPROCHE EXPERIMENTALE

A. ETABLISSEMENT D'UN MODELE MURIN

1. Production de rétrovirus contenant le cDNA de l'IL-2 ou du CD4

a) **Construction des vecteurs rétroviraux**
Le plasmide M5-néo contenant un gène de résistance à la généticine (G418) est un excellent vecteur pour l'expression de gène dans les cellules hématopoïétiques (12). Le cDNA de l'IL-2 ou du CD4 sera inséré dans le site du clonage EcoRI de M5-néo. Les séquences de polyadénylation de ces cDNA seront préalablement excisées car elles provoquent une chute drastique du taux de rétrovirus produit. L'orientation correcte du cDNA sera vérifiée en digérant le plasmide par enzyme de restriction.

b) **Production de particules rétrovirales**
Le plasmide sera transfecté dans la lignée d'emballage ecotropique GP+E86 (13) par la technique classique de co-précipitation au phosphate de calcium. Cette lignée produit, *in trans*, les protéines dérivées des gènes *gag*, *pol*, et *env* nécessaires à la production de particules rétrovirales. Le surnageant rétroviral produit par cette lignée servira à l'infection de la lignée amphotropique GP+envAm12.

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c) **Détermination du titre rétroviral**

Les cellules NIH 3T3 seront infectées avec le surnageant viral produit par cellules d'emballage GP+E86 ou GP+envAm12. Seules les NIH 3T3 ayant intégré le virus et donc le gène de résistance au G418, survivront après introduction de l'antibiotique dans le milieu. Le titre viral correspond au nombre de colonies formées par millilitre de surnageant viral.

d) **Recherche de rétrovirus recombinant infectieux**

Les surnageants de NIH 3T3 infectées par rétrovirus et sélectionnées par G418 serviront à infecter des NIH 3T3 fraîches et celles-ci seront à nouveau sélectionnées avec du G418. En l'absence de rétrovirus recombinant ces cellules ne survivront pas.

2. Etablissement du modèle animal

a) **Infection des cellules souches périphériques**

Nous utiliserons des souris C3H/HeJ car cette race a été fréquemment utilisée dans les expériences de transfert de gène par rétrovirus en raison du faible risque d'apparition de rétrovirus recombinant xénotrope chez ces animaux. Les cellules souches hématopoïétiques de ces souris seront collectées après chimiothérapie et rhG-CSF par section brachiale ou ponction cardiaque sous anesthésie générale (14). Ces cellules seront préstimulées pendant 48 heures par de l'interleukine-6 et du Stem Cell Factor pour augmenter la mise en cycle cellulaire (15) et mises ensuite durant 48 h en présence de la lignée productrice de virus préalablement irradiée. Les cellules hématopoïétiques non adhérentes seront injectées à des souris syngéniques irradiées à dose léthale.

b) **Suivi après greffe**

A court terme, nous surveillerons les paramètres hématologiques (globules blancs et plaquettes) par ponction rétro-orbitaire. La persistance du gène rétroviral dans les cellules hématopoïétiques périphériques et médullaires sera evaluée par polymerase chain reaction et son expression par Elisa pour l'IL-2 et par cytométrie de flux pour le CD4. A plus long terme, la survenue éventuelle de lymphomes suite à une stimulation du type autocrine par l'IL-2 sera évaluée par suivi clinique et examen anatomopathologique des rates et moelles des animaux sacrifiés à des temps déterminés de l'expérience.

B. DEFINITION DES CONDITIONS D'INFECTION IN VITRO DES CELLULES SOUCHES PERIPHERIQUES HUMAINES

Nous disposons d'une lignée cellulaire (PA317) produisant des rétrovirus (N2) conférant la résistance au G418 à de hauts titres et déjà utilisés dans plusieurs protocoles cliniques (16). Le titre rétroviral sera estimé et l'absence de virus recombinant sera évaluée. Des cellules souches périphériques humaines fraîchement collectées après chimiothérapie suivie de facteur de croissance seront infectées par le surnageant viral filtré car la technique de co-culture n'est pas applicable en clinique en raison des risques de contamination par les cellules murines. Les cellules souches périphériques seront donc exposées au surnageant viral durant des temps à déterminer et l'infection des cellules sera controlée par culture clonale en méthylcellulose avec ou sans G418. Les colonies résistantes seront isolées et la présence du gène de résistance au G418 sera certifiée par polymerase chain reaction.

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MECANISMES DE POLYPLOIDISATION MEGACARYOCYTAIRE DANS DES LIGNEES CELLULAIRES LEUCEMIQUES.

INTRODUCTION

Parmi les affections malignes de la moelle osseuse, les syndromes myéloprolifératifs (maladie de Vaquez, leucémie myéloïde chronique, thrombocytémie essentielle et splénomégalie myéloïde), résultent d'une transformation maligne de la cellule souche hématopoïétique et ont en commun une prolifération accrue sans blocage de maturation des trois lignées myéloïdes, une hyperuricémie, une splénomégalie, une évolution terminale fréquente en myélofibrose et en leucémie aiguë.

Le caractère monoclonal de la prolifération cellulaire affectant les trois lignées médullaires a été clairement établi par l'étude des femmes noires hétérozygotes pour les isoenzymes de la G-6-P-D dans la maladie de Vaquez, la leucémie myéloïde chronique (LMC), la thrombocytémie essentielle (TE), et la splénomégalie myéloïde (populations érythrocytaire, granulocytaire, plaquettaire, toutes porteuses de la même unique isoenzyme).

Dans les syndromes myéloprolifératifs, la lignée plaquettaire est souvent affectée soit par les anomalies des fonctions plaquettaires soit par le nombre excessif des plaquettes. Cependant, il existe une hétérogénéité au sein des différents syndromes. En effet, alors que dans la leucémie myéloïde chronique, le volume mégacaryocytaire est petit ou normal, dans la thrombocytémie essentielle, la caractéristique médullaire est une augmentation de la ploïdie mégacaryocytaire.

Sur base de l'étude morphométrique des mégacaryocytes de souris, Harker a montré en 1968 une corrélation inverse entre le volume mégacaryocytaire moyen et la concentration plaquettaire (1,2). Après induction d'une thrombopénie, le volume mégacaryocytaire moyen augmente. Par contre, suite à l'hypertransfusion de plaquettes, la taille des mégacaryocytes diminue. Cette régulation est réalisée par une substance humorale appelée "thrombopoïétine" dont la nature reste à découvrir. Plusieurs cytokines ont une action thrombopoïétique (IL-6, IL-11, LIF ...). Par ailleurs, l'oncogène mpl issu d'un virus responsable chez la souris d'un syndrome myéloprolifératif avec fibrose médullaire code pour une protéine présentant des ressemblances avec certains membres de la famille des récepteurs de croissance hématopoïétiques. L'homologue humain de cet oncogène a été cloné (3). Les lignées mégacaryocytaires connues expriment intensément cet oncogène suggérant qu'il code pour un récepteur à un facteur très spécifique de la mégacaryocytopoïèse (4). Chez l'homme une corrélation similaire a été observée en condition normale. En pathologie, cette corrélation entre le volume mégacaryocytaire moyen et la concentration plaquettaire se vérifie d'une façon assez surprenante dans la leucémie myéloïde chronique alors que dans la thrombocytémie essentielle le volume mégacaryocytaire moyen élevé semble échapper au contrôle thrombopoïétique. Ces données ont été confirmées en cytofluorométrie en flux par Tomer (5) ainsi que par notre équipe.

Il est impossible d'obtenir une lignée continue à partir de cellules normales. La leucémie myéloïde chronique est particulièrement intéressante puisqu'elle présente des troubles de prolifération couplée à une régulation normale mégacaryocytaire. Nous avons établi dans notre laboratoire quatre lignées nouvelles issues de deux patients atteints de leucémie myéloïde chronique (lignées DB et DL) et de deux patients atteints de thrombocytémie essentielle (lignées AA et DD). L'étude morphométrique des mégacaryocytes de ces patients est en accord avec la théorie de Harker. Ces lignées sont établies et repiquées depuis plusieurs mois dans un milieu supplémenté avec 10% de sérum de veau foetal.

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Par l'étude de ces lignées cellulaires issues de deux affections malignes prolifératives ou le phénomène de polyploïdisation est normal (LMC) ou anormal (TE), nous proposons d'étudier les anomalies du contrôle de l'endomitose mégacaryocytaire.

BUT DU TRAVAIL

Notre projet a pour but de déterminer les anomalies de la polyploïdisation entraînant l'hyperplaquettose de la TE à partir des lignées continues humaines que nous avons établies. La comparaison se fera avec nos lignées continues issues de patients atteints de LMC où le contrôle de la polyploïdisation mégacaryocytaire est normale. Nous avons montré que l'absence de polymérisation de l'actine induit la polyploïdisation des mégacaryocytes. Les esters de phorbol et certaines cytokines augmentent la ploïdie mégacaryocytaire, nous voulons démontrer que leur action sur ces lignées s'exerce sur le métabolisme de l'actine.

APPROCHE EXPERIMENTALE

1. **Développement de nouvelles lignées continues mégacaryocytaires**
   Nous avons donc établi des lignées continues à partir de moelle de patients atteints de LMC et de thrombocytémie essentielle. D'autres affections malignes de la moelle osseuse s'accompagnent de perturbation de la mégacaryocytopoïèse (la maladie de Vaquez, la splénomégalie myéloïde, les myélodysplasies et certaines leucémies aigues à caractère mégacaryocytaire). Parallèlement à la caractérisation des lignées déjà établies, nous poursuivrons le développement d'autres lignées au fur et à mesure des opportunités de la clinique.

2. **Caractérisation des lignées établies**

a. **Cytologie**
Les quatres lignées seront analysées du point de vue cytologique par la coloration classique de May-Grünwald Giemsa (MGG). Ensuite, la présence de caractéristiques mégacaryocytaires telles que la présence de membranes de démarcation, des granules denses et granules α ainsi que le contenu en péroxydase plaquettaire seront étudiés par microscopie électronique.

b. **Cytochimie**
Les examens cytochimiques nous permettront de détecter en microscopie optique l'activité de la myélopéroxydase, des phosphatases acides, des estérases (alpha-naphtyl, alpha-naphtyl-butyrate, chloro-naphtyl-ASD acétate, estérases) ainsi que la positivité pour la réaction de PAS (Periodic Acid Schiff).

c. **Immunologie**
La mesure de ploïdie (coloration à l'iodure de propidium) est réalisée en cytofluorométrie en flux. Le contenu protéique pourra être simultanément quantifié par un immunomarquage direct à la fluorescéine. La cytofluorométrie en flux nous permettra de mesurer l'expression des protéines plaquettaires. C'est ainsi que notre étude portera sur la mise en évidence des glycoprotéines membranaires : la GPIIb/IIIa et la GPIb ainsi que sur les facteurs VIII et de von Willebrand. Par ailleurs, les contenus de granules alpha en PDGF, fibrinogène, facteurs de von Willebrand, β-TG et PF4 seront mesurés de même que le contenu des granules denses.

d. **Taille et distribution de ploïdie à l'étape basal**
Par morphométrie (Harker, 1968), nous pourrons estimer la taille des cellules et, par cytofluorométrie, la taille et la ploïdie des noyaux cellulaires.

e. **Caractéristique génétique**
Une analyse cytogénétique révèlera le caryotype de nos cellules ainsi nous confirmerons la nature maligne de nos cellules et l'absence d'anomalie supplémentaire. Les deux patients atteints de LMC et dont les lignées sont issues ont un réarrangement du bcr.

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3. **Caractérisation en culture et étude de la polyploïdisation**

a. **Taux de prolifération en culture**

Nous établirons le taux de prolifération en culture en calculant l'augmentation cellulaire en fonction du temps et en mesurant le taux d'incorporation de la thymidine tritiée.

b. **Culture sans sérum**

Mises en présence d'un milieu synthétique de base, beaucoup de cellules ne font que survivre. Le déclenchement de la division cellulaire n'est possible qu'en présence d'un certain nombre de facteurs mitogènes, le plus souvent fourni par le sérum. Cependant, le sérum a plusieurs inconvénients car il se présente sous forme de mixture très complexe dont les composants sont mal définis. C'est pourquoi, il s'avère intéressant de remplacer le sérum par un mélange connu de facteurs de croissance (milieu défini).

c. **Réponse aux cytokines**

Parmi les cytokines, l'IL-6, l'IL-11, le LIF, l'érythropoïétine et l'IL-3 ont la capacité d'augmenter la ploïdie des mégacaryocytes normaux. Nous étudierons la ploïdie des cellules de nos lignées suite à l'adjonction de ces facteurs. Une éventuelle synergie sera étudiée par l'addition de combinaison de ces facteurs.

Nous étudierons l'expression de l'homologue v-mpl dans les lignées que nous avons établies et sa modulation par les facteurs de croissance. Les oligonucléotides antisens seront utilisés pour définir le rôle de cette nouvelle molécule dans la lignée mégacaryocytaire (ploïdie en cytofluorométrie en flux). Cette technologie sera aussi appliquée à l'étude de la modulation de l'expression des gènes codant pour les récepteurs aux cytokines connus pour leur action stimulante de l'endomitose (IL-6R, LIF-R, IL-11-R...).

4. **Etude de l'actine**

Nos études chez la souris suggèrent que l'absence de polymérisation de l'actine entraîne l'endomitose mégacaryocytaire (6). Nous avons confirmé ces données chez l'homme par l'étude de lignées leucémiques à caractéristique mégacaryocytaire (DAMI, MEG01, HEL, K562). Nous avons remarqué après quatre jours de culture une augmentation de la taille et de la ploïdie de ces cellules suite à l'adjonction d'ester de phorbol (stimulant non spécifique des récepteurs aux facteurs de croissance) et/ou de cytochalasine B (inhibiteur de la polymérisation de l'actine). Ces expériences seront également réalisées sur nos propres lignées. Le rôle physiologique de l'actine dans l'induction de la polyploïdisation doit encore être démontré. Dans ce but, nous comptons mesurer l'actine sous ses différentes formes :

a. **Mesure globale au cytofluoromètre en flux.**

Nous avons mis au point la mesure globable de l'actine sur des populations lymphocytaires par marquage à l'aide d'un anticorps dirigé contre l'actine. Les quantités globales d'actine seront mesurées sur les quatres lignées mégacaryocytaires à notre disposition avant et après stimulation au PMA et à la cytochalasine B. Une corrélation avec la ploïdie de ces cellules pourra être effectuée par marquage concomittant des cellules à l'iodure de propidium. Dans ce cas, trois possibilités peuvent être envisagées. Tout d'abord, une absence d'augmentation de l'actine permettrait de postuler que la plus grande partie de l'actine est impliquée. Ensuite, une augmentation "saltatoire" ou progressive suggèrerait qu'une faible partie seulement de l'actine serait impliquée dans l'endoréduplication.

b. **Mesure qualitative de l'actine G et de l'actine F**

L'actine se présente sous deux formes : l'actine G monomérique et soluble et l'actine F polymérisée et insoluble. L'actine G tout comme l'actine F ont la capacité de se lier à toute une série de ligand. La profiline se lie à l'actine G et gène la polymérisation.

La gelsoline rompt les filaments d'actine F et se lie à une des extrémités. Ces deux protéines rendent compte de la structure de la cellule. La tropomyosine se lie latéralement le long du filament et la myosine se déplace le long de celui-ci. Ce sont elles qui assurent la motilité de la cellule. Enfin, la villine et l'alpha-actinine connectent les filaments entre eux formant ainsi les trames ou réseaux.

La concentration d'actine est relativement constante dans toutes les cellules. C'est d'ailleurs pour cette raison qu'elle a été utilisée comme contrôle de l'expression génique d'autres molécules. Quelques anticorps monoclonaux reconnaissent différents épitopes de l'actine. Malheureusement, ils ne permettent pas réellement de déceler les sites de liaison aux différents ligands et dès lors de mettre en évidence les différents types d'actine. C'est par

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marquage fluorescent que nous pourrons mettre en évidence dans l'endomitose le type d'actine et donc la fonction perturbée (motilité, structure par exemple,...). De plus, la phalloïdine qui se lie spécifiquement au filament d'actine permettra d'analyser le processus dynamique de la formation d'actine. Dès lors de façon à mettre en évidence le type précis d'actine, nous emploierons le tri cellulaire au cytofluoromètre en flux (Coulter Elite) sur les lignées ainsi que sur les cellules normales. Cette technique a déjà été mise au point dans notre laboratoire. Nous avons déjà séparé des mégacaryocytes en diverses phases mitotiques (2N et > 2 N). Après tri de ces cellules en fonction des classes de ploïdie, nous pourrons par électrophorèse déterminer les modifications quantitatives et qualitatives de l'actine.

c. **Modification de l'expression du gène de l'actine**

L'expression du gène de l'actine est un phénomène modulable. Les agents adrénergiques et l'héparine conduisent à la synthèse d'actine (7,8,9,10). Nous testerons leurs effets sur l'induction de polyploïdisation de nos lignées cellulaires. Certains facteurs de transcription contrôlent la synthèse d'actine. Par addition d'oligonucléotides antisens au milieu de culture, nous bloquerons ces facteurs de transcription et étudierons leurs effets sur la ploïdie des cellules des lignées en culture.

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ACCEPTED FOR PUBLICATION
IN CANCER RESEARCH

TRANSCRIPTION OF GENES ENCODING GM-CSF, IL-3 AND IL-6 RECEPTORS AND LACK OF PROLIFERATIVE RESPONSE TO EXOGENOUS CYTOKINES IN NON-HEMATOPOIETIC HUMAN MALIGNANT CELL LINES[1]

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Running Title : Cytokine Receptor Gene Transcription in Malignant Cells.

Key Words : Cytokine receptors, cytokine stimulation, gene transcription, malignant cell lines, tumor growth.

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3 The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; PCR, polymerase chain reaction; R, receptor; MEM, modified Eagle's medium; FCS, fetal calf serum.

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ABSTRACT

Studies in recent years have suggested that human tumor cell lines are capable of responding in vitro to hematopoietic growth factors. In the present study, we investigate the transcription of the α and β subunits of GM-CSF receptor (GM-CSFR), the α and β subunits of IL-3 receptor (IL-3R), the single subunit of IL-6 receptor (IL-6R) and its associated gp130 transduction protein by PCR amplification of reverse transcribed cellular mRNA in 34 malignant cell lines derived from a variety of histologic cell types. mRNA for only a single subunit polypeptide was found in a significant minority of cell lines (23%), while in 20% both the α and β subunits of either the GM-CSFR or the IL-3R were detected, distributed among a number of different histological cell types. Transcription of the gene encoding the IL-6R was found in 38% of cell lines, and all lines transcribed the gp130 transduction protein, consistent with previous observations on the ubiquity of that polypeptide. In order to test the in vitro effect of exogenously added growth factors on those malignant cell lines transcribing complete cytokine receptor, either GM-CSF, IL-3 or IL-6 was added in therapeutic concentrations (20-500 ng/ml) and cellular proliferation measured by incorporation of [^{3}H]-thymidine. No stimulation was seen at either 3 and 6 days of culture. Production of cytokine by these cell lines was investigated at the level of transcription and by assay of peptide product. None transcribed mRNA for either GM-CSF or IL-3, while 5 of six (STD, DOZ, ADE, Hep-2 and Detroit) expressed IL-6 mRNA. Of these latter, 2 (lines ADE and Hep-2) produced IL-6 as determined by bioassay, while none produced GM-CSF or IL-3 by ELISA. This suggests that in the case of GM-CSF and IL-3, failure to proliferate on addition of cytokine is not due to the prior presence of endogenous production. In contrast, at least a subset of malignant cell lines may involve a closed IL-6 autocrine loop saturating cell surface sites. These findings suggest that the ability to transcribe the genes encoding cytokine receptor is alone insufficient to render cells cytokine-responsive, and that malignant cells may lack the cellular machinery for cytokine-induced proliferation. This in turn suggests that therapeutic administration of either GM-CSF, IL-3 or IL-6 may involve no additional risk of tumor regrowth in vivo.

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INTRODUCTION

GM-CSF[3] and IL-3 are potent hematopoietic growth factors stimulating the proliferation and differentiation of both multipotent stem cells and lineage-committed hematopoietic cells (1,2). The transmembrane cell surface receptors which bind these growth factors and transduce their signal intracellularly have been cloned and characterized in recent years. The human GM-CSF receptor (GM-CSFR) is composed of two subunits, a low affinity alpha subunit specific for the GM-CSFR (3) and a beta subunit, which it shares in common with the IL-3 receptor (4). Together, these make up the 45 kDa receptor, and their coexpression results in high affinity GM-CSF binding (5). The IL-3 receptor (IL3R), likewise composed of two subunits, the common beta subunit and a recently cloned specific alpha subunit, has a combined molecular weight of 120 kDa (4,5). Interleukin-6 (IL-6) is a pleiotropic cytokine involved in immune and inflammatory processes in addition to its role in hematopoietic maturation (6). Its receptor (IL-6R), composed of a single unit with a molecular weight of 80 kDa, and an associated gp130 transduction protein have been cloned and characterized (7-9).

The influence of hematopoietic growth factors has recently been examined in a variety of human solid tumor cell lines, including those derived from small cell lung cancer, from melanoma, as well as from renal, colon gastric and ovarian carcinomas. Several reports suggest that GM-CSF, IL-3, and IL-6 might be capable of stimulating the growth of some of these malignant cell lines in vitro (10-21), although discrepancies have been found in comparing different studies of the same histologic tumor type and even among tumor cell lines derived from the very same source. These apparent inconsistencies likely arise from the biological heterogeneity of the tumors studied. What emerges, however, is the possibility that malignancies, or at least a subset of malignant cells, may owe part of their capacity to proliferate to stimulation by cytokines. Moreover, the stimulation may result from either a paracrine effect of cytokine produced by a source other than the tumor itself, or from a self-sustaining autocrine loop of tumor-produced cytokines (22-24).

Because of the increasing use of hematopoietic growth factors in clinical settings following chemotherapy and autologous bone marrow transplantation (25), there is concern that these

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stimulating factors, while promoting re-population of the bone marrow, might at the same time induce regrowth of residual tumor. In this report, we analyze the transcription of the genes encoding the GM-CSF, IL-3, and IL-6 receptors in 34 cell lines derived from a variety of human malignancies. RNA message for cytokine receptors was found to be transcribed to a greater degree than previously reported. However, despite evidence for transcription and expression of growth factor receptors by tumor cells, no indication was found that this results in cellular proliferation in vitro when the corresponding factors are exogenously added in a range of concentrations analogous to those administered therapeutically. More than just the presence of cell surface receptors may be required for a stimulatory response to cytokines; Failure to proliferate may be due to either an inability of tumor cells to bind cytokines in adequate concentrations or to an inability to properly transduce signal from the cell membrane to the cytosol. These in vitro findings imply that in vivo therapeutic administration of hematopoietic growth factors may entail no proliferation of tumor cells and no added risk of tumor recurrence clinically.

MATERIALS AND METHODS

Cell lines. Small cell lung cancer cell lines (OC1, OC2, OC3, FRA, STO, GIL, THO, BAT, DEV, VHE, FIC, DOZ) were established in our laboratory except for two cell lines (H69 and H128) kindly provided by the National Cancer Institute (Bethesda, MD), and were cultured in RPMI-HITES medium (26). Melanoma cell lines (DES, G43, WO, RENI, ADE) were developed in our laboratory and maintained in Dulbecco's medium with 20% Hepes, 4.5% glucose and 10% FCS. Head and neck carcinoma cell lines (Fadu, KB, Hep-2, Detroit 562, RPMI 2650) were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in MEM with non-essential amino acids and 10% FCS. Ovarian neoplastic cell lines (AZ 224, AZ 382, AZ 364, AZ 303) obtained from Dr. J. De Grève (Division of Medical Genetics, Vrije Universiteit, Brussels) and prostatic carcinoma (DU145) cell line purchased from the ATCC were cultured in RPMI 1640 supplemented with 10% FCS. Two gastric cancer cell lines from the ATCC (AGS and KATO III) were cultured in Ham's F12 and RPMI 1640 media respectively. Finally, three kidney carcinoma cell lines obtained from the ATCC (CAKI I, CAKI II, A498) were maintained in

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McCoy's or MEM media. HL60 cells (a human promyelocytic cell line) (27), KG1 cells (28) and CESS cells (an EBV-transformed B cell line) (29) were used as positive controls.

PCR amplification and analysis of product. Total RNA was extracted from $5x10^6$ cells either using the phenol extraction method (30) or the guanidium method (31). cDNA was synthesized using 1 ug of total RNA with reverse transcriptase (BRL, Gaithersburg, MD), and random hexanucleotides (Pharmacia, Milwaukee, WI) in a 20 μl volume containing final concentrations of 50 mM Tris pH 8.3, 20 mM KCl, 10 mM $MgCl_2$, 5 mM DTT and 1 mM of each dNTP. 5 μl of the reverse transcriptase reaction was used for PCR amplification in a 50 μl volume containing 15 picomoles of primers, 1 nanomole of each dNTP, and 2.5 units of Taq DNA polymerase (BRL) in a final concentration of 50 mM KCl, 10 mM Tris-HCl pH 8.3, and 2.5 mM $MgCl_2$. Reaction mixtures were subjected to 25 successive cycles consisting of heat denaturation (94°C, 1.5 min), annealing (55°C for 2 min for GM-CSFR alpha, GM-CSFR beta, IL-6R, IL-3, and IL-6, 60°C for IL-3R alpha, GM-CSF, and 65°C for human beta actin) and primer extension (72°C for 3 min). PCR products were size fractionated in 2% agarose gels, and blotted onto Genescreen Plus nylone membranes (Dupont NEN, Boston, MA). The blots were hybridized with gene-specific oligonucleotides probes internal to the paired PCR primers used in generating each product. Oligonucleotides were end-labelled by T4 polynucleotide kinase and gamma-[^{32}P] ATP (32). The sequences of each set of PCR primers and their corresponding gene-specific internal oligonucleotide probes are depicted in Table 1 (first citation of Table 1). Conditions of prehybridization, hybridization and washing were performed as previously described (32) at the temperatures indicated in Table 1.

First citation of Table 1

Assay for endogeneous cytokine production by malignant cells. Supernatants of malignant cell lines of at least 3 days growth were assayed for the presence of GM-CSF and IL-3 in 96 well-plates by ELISA as recommended by the manufacturer (Amersham International, UK). IL-6 activity was measured using the IL-6-dependent murine hybridoma 7TD1 as previously described (33).

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Cellular Proliferation Assay. Cells were cultured, in triplicate, in 100 μl of their respective medium containing FCS at a concentration lower than that used in the original cultures (3%) in 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA) at a concentration of 10^5 cells per ml. Human recombinant growth factors rGM-CSF, rIL-3 and rIL-6 (provided by Sandoz, Basel, Switzerland) were added at three different concentrations (20, 100, 500 ng/ml) to each set of triplicate wells at the initiation of the culture. After 3 and 6 days of culture, 0.5 uCi of [^{3}H]-thymidine (Dupont NEN) was added to each well and cells were harvested 24 hours later.

Statistical analysis. Values are expressed as the mean and ± standard error of the mean (SEM). The significance of differences between groups was calculated using the Student-Newman-Keuls test.

RESULTS

PCR amplification products of the cytokine receptor gene transcripts under study are depicted in Table 1. As indicated, the expected lengths of the gene transcripts were 530 bp for GM-CSFRα, 442 bp for IL-3Rα, 316 bp for the common β chain, 306 bp for IL-6R, 322 bp for GM-CSF, 344 bp for IL-3, and 459 bp for IL-6. In addition, the PCR primers corresponding to the IL-6 signal transducer yielded a 759 bp product and those for actin yielded a 519 bp product. Amplification with the actin primers confirmed the presence of intact cDNA in each sample. Hybridization of each PCR product to its corresponding gene-specific internal oligonucleotide probe confirmed the specificity of the amplifications.

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Results of cytokine receptor gene expression are presented in Table 2 (first citation of Table 2). Transcription of the gene encoding only a single subunit of the GM-CSFR or IL-3R was detected in a significant minority of cell lines (8/34, 23%). The alpha subunit of GM-CSFR was detected alone in 6/34 of the lines while the alpha subunit of IL-3R alone was not found in any. Both alpha subunits were identified in 2/34 cell lines. The beta subunit, common to both the GM-CSFR and IL-3R, was found as the sole transcript in 1/34 cell lines. Expression of a single

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subunit, or of only alpha subunits of both GM-CSFR and IL-3R, without concomitant production of beta subunits confers only low affinity binding to their respective cytokines. Simultaneous expression of both the alpha and beta subunits of either the GM-CSF or the IL-3 receptor was seen in 7/34 (20%) of the cell lines. Of these, transcripts of the two GM-CSFR subunits alone were detected in 1 and those of IL-3 in 2, while in 4 cell lines, both subunits of both receptors were identified. These included 2 small cell carcinoma cell lines, 1 melanoma cell line, 2 head and neck cancer cell lines, 1 ovarian carcinoma, and 1 renal carcinoma cell line. Transcription of the gene encoding the IL-6R was found in 13/34 (38%) cell lines including 2 small cell lung cancers, 2 melanomas, 4 head and neck tumors, 1 prostatic carcinoma, 1 gastric carcinoma, and 3 renal carcinomas. The expression of the IL-6 signal transducer, gp130, was expressed by all cell lines regardless of the concomitant transcription of the other cytokine receptors, including IL-6R.

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Characteristic results of PCR amplification of the panel of tumor cells are shown in Figure 1 (first citation of Fig.1). As indicated, a relatively high frequency of transcription of cytokine receptor genes was found. Amplification of even minute amounts of reverse-transcribed cDNA could conceivably yield false positive results. For this reason, we diluted the reverse-transcribed cDNA serving as template by 1:100 in those cell lines yielding initial positive PCR products. The diluted material was then amplified and the product quantitatively compared to that obtained from the PCR product using as template undiluted cDNA from the positive control cell lines by hybridizing to the corresponding internal oligonucleotide probe (not shown). Simultaneously run appropriate negative controls assured that positive samples did not result from contamination.

To test the in vitro proliferative effect of exogenously added growth factors on malignant cells, those cell lines expressing both subunits of either GM-CSFR, IL-3R, or those transcribing the single unit of IL-6R were cultured with their appropriate cytokine in a concentration range of 20 ng/ml to 500 ng/ml and stimulation was measured by incorporation of [^{3}H]-thymidine. No stimulatory effect as compared to the control culture was seen at any of the concentrations for either GM-CSF, IL-3 or IL-6 at either 3 or 6 days of culture (Table 3) (first citation of Table 3). The lower concentration of GM-CSF and IL-3 actually yielded diminished incorporation [^{3}H]-thymidine in cell line STO and DOZ suggesting that antiproliferative effect at those concentration

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cannot be definitively ruled out.

By ELISA, neither GM-CSF nor IL-3 were detected in the supernatants of the cell line used in the proliferative assays. IL-6, as measured by bioassay with the IL-6-dependent murine hybridoma 7TD1 (33) is absent in the supernantants of lines ST0, D0Z and Detroit, while present in lines ADE and Hep-2 at 200 ng/ml and 225 ng/ml respectively.

Analysis by PCR amplification of the expression of the cytokine gene transcription corroborates these results. Transcripts of neither GM-CSF and IL-3 were not found in any cell line studied. However, transcription of the gene encoding IL-6 was detected in most cell lines from a variety of histologic origins (Table 4) (first citation of Table 4).

Fi st citation of Table 4

DISCUSSION

Hematopoietic growth factors have won an established place in the prevention and treatment of the hematologic complications induced by cancer chemotherapy. However, a number of reports have suggested that growth factors might also induce the in vitro proliferation of non-hematologic malignant cell lines or at least a subset of tumor cell lines (10-21). Presumably, the inappropriate presence of cell surface receptors for cytokines on tumor cells contibutes to the growth deregulation characteristic of malignancies. The use of growth factors, then, might facilitate the progression of the residual disease or, depending of the factor involved, might conceivably induce tumor cell differentiation as well.

In the present study, on examining the transcription of the genes encoding receptors for growth factors, we observed a relatively frequent expression of both subunits of either the GM-CSFR or the IL-3R (20%) as well as of the single subunit of IL-6R (38%). These included cell lines derived from a variety of malignant histologic types (Table I). Expression of the IL-6 signal transducer, gp130, was detected in all cell lines, even in those not transcribing the IL-6 receptor gene itself. This is consistent with previous observations on the ubiquity of gp130 expression (8). The ability of non-hematopoietic tumor cells to transcribe mRNA for receptors of normal hematopoietic growth factors suggests that the genes encoding those cell surface peptides are among the dysregulated genes that may be a marker of or even possibly causally linked to

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malignant proliferation. Significantly, the fact that some malignant cells dysregulate the gene encoding a single receptor subunit, while others dysregulate two genes points to the heterogeneity of tumors, even among those of the same histologic type (Table 2) regardless whether the receptor is functional or not. Further biological heterogeneity may be introduced by differential abilities to translate peptide products and assemble them on the cell surface as structurally and functionally the same receptor as present on normal hematopoietic cells. This forms the basis of further investigations (T. Guillaume et al, manuscript in preparation).

The sensitivity of PCR amplification could yield false positive results and thereby overestimate transcription of cytokine receptor genes. Generation of PCR product is geometrically proportional to the amount of template available for amplification (39). With the quantitative technique used in this study, even 1:100 dilutions of the reverse-transcribed cDNAs serving as PCR template yielded products detectable at the same level as detected with undiluted cDNA derived from the hematopoietic cell line serving as positive control. Had there been a systematic laboratory contaminant of samples actually negative for the receptor, one might have expected the dilutions to give product yields of varying intensity in quantitative PCR, depending on their degree of contamination. Furthermore, simultaneously run side-by-side negative controls were consistently negative. In fact, a majority of the simultaneously amplified malignant cell lines studied were negative for transcription of complete two-sub-unit cytokine receptors in the very sensitive oligonucleotide probe hybridizations used here, further arguing against indiscriminant, artifactual amplification. Basal levels of gene transcription of tissue-specific genes in non-specific cells, a phenomena termed illegitimate transcription, has been demonstrated by PCR amplification in a number of genes including β globin, Factor VIIIc, aldolase A, and anti-Müllerian hormone (40). It has not yet been demonstrated if it is a universal phenomena for all human genes. Even if true for the genes encoding cytokine receptors, it is reasonable to assume that as in the case of other tissue-specific gene, only those transcribed at a significant level may potentially produce biologically-active products. In the case of cytokine receptors, significant levels are those comparable to those in established cell line known to be cytokine-responsive which we have used as positive controls.

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Cell lines transcribing the complete receptors for either GM-CSF, IL-3 or IL-6 were examined in medium containing low concentration of FCS (3%) in the presence of a wide range of concentrations of cytokines, from 20 to 500 ng/ml, known to stimulate hematopoietic progenitor cell proliferation in vitro. No proliferative enhancement as measured by incorporation of [^{3}H]-thymidine could be detected at either 3 or 6 days of culture. In addition, at the lower concentrations of GM-CSF and IL-3 (20 and 100 ng/ml) decreased in [^{3}H]-thymidine incorporation was seen in cell lines STO and DOZ, so that an antiproliferative effect, at least on those two lines, cannot be entirely ruled out. These findings suggest that the growth factors neither induce previously non-clonogenic cells to clonal growth nor increase the size of existing clones. Since the cells themselves produce neither GM-CSF or IL-3, as determined both by the absence of gene transcription (Table 4) as well as by ELISA, the lack of response to exogenous GM-CSF or IL-3 is not due to occupation of all available cell surface receptor sites by endogenously produced factors. However, lines ST0, DOZ, ADE, Hep-2 and Detroit do transcribe the IL-6 gene (Table 4). Despite this, IL-6 activity, as measured by bioassay is absent in 3 (ST0, DOZ and Detroit) of these 5 lines. Since the bioassay requires intact cytokine, IL-6 gene transcription divorced from elaboration of the functional peptide product suggests either dysregulated translation, decreased cytoplasmic half-life, or a non-functional cytokine. As in IL-3 and GM-CSF, the absence of IL-6 production militates against the presence of an autocrine loop as an explanation for the lack of proliferative effect of exogenously added IL-6 in those lines. However, cell lines ADE and Hep-2 do produce detectable levels of IL-6 and their IL-6 receptor sites may already be occupied by endogenous cytokine. Conceivably, even small amounts of IL-6 could occupy sufficient surface receptor sites to block added IL-6 even in cell lines evidencing IL-6 mRNA transcription but no IL-6 production. However, in such a case, a disparity would remain between the strongly positive results on quantitative PCR determinations of mRNA and even a putatively low level of IL-6 production, below even detection by bioassay. Furthermore, the apparent discrepancy between cellular transcription of message encoding the cytokine receptors (Table 2) and the absence of proliferation in vitro on addition of cytokines (Table 3) suggests ineffective receptor peptide assembly despite production of full-length RNA message.

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Alternatively, low density of cytokine receptor peptides on the tumor cell surface may preclude effective binding of cytokine, or, even in the presence of adequate cytokine binding, the cell may lack the appropriate machinery for signal transduction.

Previous studies of the in vitro proliferative effects of GM-CSF on tumor cells have not been conclusive. While stimulations of fresh solid tumor biopsies including from breast, colon, lung, ovarian carcinomas as well as melanomas have been almost uniformly negative (13,14) proliferative responses to GM-CSF have been reported in established tumor cell lines, although variable results have been recorded between different laboratories, at times using the very same cell lines. That tumors of the same histopathology but of different sources might give variable results is not altogether surprising; malignancies, even of the same cell type, are by their nature biologically heterogeneous. In addition, heterogeneity of continually passed cell lines, even from the very same original source, can probably account for a great deal of apparently inconsistent in vitro results. The advantage of the present report is that cell lines used both in the cytokine receptor gene transcription study and in the proliferation assays include lines developed by us which have not undergone continual passage, and therefore are more likely to reflect in their biological activity the original tumors from which they were derived.

Studies regarding the in vitro effects of IL-3 on malignant growth have been less extensive than those for GM-CSF. Evidence exists, in a limited study, that IL-3 enhances the growth of pancreatic and gastric carcinoma cell lines and was additive to the stimulatory effect of GM-CSF (17). Likewise, IL-3 has been found to be stimulatory to a single colon carcinoma cell (11), and small cell lung carcinoma cell lines (18). In contrast to GM-CSF, IL-3 has also be found to be stimulatory in a minority (23%) of primary tumor biopsies in a tumor cloning assay (15).

Perhaps the most consistent observations have been made with IL-6. By immunochemical staining, IL-6 has been found to be present in a wide variety of solid tumors including colon, ovarian, and breast carcinomas as well as in Kaposi's sarcoma (41), in squamous cell carcinoma and in soft tissue sarcomas (42). However, such staining is unable to differentiate between endogenously produced tumor IL-6 and mere adhesion of cytokine produced elsewhere. On the other hand, renal carcinoma cell lines have been shown to both produce and proliferate in

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response to IL-6 and demonstrate growth arrest in the presence of anti-IL6 antibodies (43, 44). Expression of both IL-6R and IL-6 has been demonstrated in prostatic carcinoma (21), and while ovarian carcinoma cell lines have been seen to have both constitutional and inducible production of IL-6 (22, 42), addition of exogeneous IL-6 did not affect cell proliferation (45). It is unclear whether this means IL-6 has no growth effect on the tumor cells or whether endogenously produced IL-6 already occupied all available receptor sites. Similarly, elevated levels of IL-6 have been detected in human hepatic carcinoma cell lines (46).

In the present study, the presence of either an exterior cytokine paracrine effect or of self-sustaining autocrine loop in those malignant cells transcribing cytokine receptors is unlikely, given both the lack of cytokine production by the malignant cell themselves as measured by either ELISA or bioassay and the absence of proliferative response to exogeneously administered cytokines in vitro as measured by incorporation of [^{3}H]-thymidine. By design, the proliferative studies were carried out in flat-bottomed wells to avoid cellular confluence that might lead to contact inhibition and absence of confluence was ascertained visually. Proliferative studies depend on the cells being in active growth phase. The concentration of cells (10^5/ml or 10^4/100 μl well), the range of added cytokines (20-500 ng/ml) and the incubation period (3 and 6 days) are in keeping with previous cellular proliferative studies (10,16,17,18,19). In most other studies, as in the present one, growth factors were added only at the beginning of the culture period; only in the study of Dippold et al. (17) were growth factors added initially and every other day. Here, as in all published reports, conclusions regarding cellular proliferation are limited to the conditions used Even if present, an in vitro stimulatory effect of cytokines on malignant cells would not necessarily translate into enhanced cell growth in vivo. Berdel et al (47) have demonstrated that two malignant cell lines, respond to exogeneous GM-CSF and IL-3 by enhanced growth in vitro. However, when xenotransplanted in nude mice, the two malignant cells lines were not induced by those same growth factors administered in vivo to the mice hosts. In vitro growth stimulation may in general not be easily extrapolatable to in vivo tumors. Apparent inconsistencies between the two might arise from differing pharmacokinetics of cytokines in vitro and in vivo. In addition, there is inherent heterogeneity of in vivo tumors even as compared to in vitro tumor cell lines,

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and the same clonogenic subpopulation present in vitro may not be the one predominating in vivo. Furthermore, apart from any direct effect of cytokine on malignant cells, growth factors can independently promote antitumor toxicity in vivo. The systemic administration of IL-6 in vivo has been shown to mediate reductions in pulmonary and hepatic metastases in syngeneic murine tumors (48,49) which appears to be due to in vivo generation of tumor-specific cytotoxic T cells at the tumor site. In fact, unlike the other cytokines studied here, IL-6 has been seen to directly inhibit malignant cells : In vitro, IL-6 has been shown to suppress the proliferation of human breast carcinoma and lymphoma cell lines (50).

In vitro experimental evidence gathered to date has suggested the possibility of in vitro stimulation of at least a subgroup of malignant cell types by cytokines. Our own findings confirm the ability of a significant minority of tumor cells to transcribe the genes encoding cell surface cytokine receptors. However, the lack of in vitro cellular proliferation to exogenously provided growth factors, together with the absence of endogenous production of cytokines suggests that even though possessing the ability to transcribe message for cytokine receptors, the malignant cell line studied here, derived from a variety histologic cell types, lack the cellular machinery necessary to respond to growth factors. This in turn suggests that in vivo therapeutic administration of cytokines in patients with solid tumors may entail no any additional risk of stimulating tumor regrowth.

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Fig. 1. PCR amplification of genes encoding cytokine receptors. cDNA was prepared by reverse transcription of total cellular RNA derived from malignant cell lines. The product was used as template for 25 cycles of PCR amplification with cytokine receptor gene-specific primers (Table 1). PCR products were size fractionated on 2% agarose gels, blotted onto nylon membranes and hybridized to internal oligonucleotide probes end-labelled with T4 polynucleotide kinase and gamma ^{32}[P]-ATP. Molecular weights are indicated to the right.

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TABLE 1 Primers used for [illegible] amplification and internal oligonucleotide probes for detection of PCR products

PCR PRIMERS			LENGTH OF AMPLIFICATION PRODUCT	REFERENCE
GM-CSFR α	sense	5'-CTTCTCTCTGACCAGCA-3'	530 bp	[3]
	anti-sense	5'-ACATGGGTTCCTGAGTC-3'		
GM-CSFR β	sense	5'-TCACTCCACTCGCTCCAGAT-3'	316 bp	[5]
	anti-sense	5'-AATACATCGTCTCTGTTCAG-3'		
IL-3R α	sense	5'-ATGTGACCGATATCGAGTGTG-3'	442 bp	[4]
	anti-sense	5'-GATACCGAAGGCTGCGCTCCT-3'		
IL-6R	sense	5'-TCCACGACTCTGGAAACTAT-3'	306 bp	[7]
	anti-sense	5'-ACTATGTAGAAAGAGCTGTC-3		
gp130	sense	5'-ATTCCTAAGGAGCAATATAC-3'	759 bp	[9]
	anti-sense	5'-TGGTCTATCTTCATAGGTGT-3'		
GM-CSF	sense	5'-TGGCCTGCAGCATCTCTGCA-3'	322 bp	[34]
	anti-sense	5'-GTGATAATCTGGGTTGCACA-3'		
IL-3	sense	5'-AGACAAGGTCCTTGAAGACA-3'	344 bp	[35]
	anti-sense	5'-CTCAAGGGTTTTCAGATAGA-3'		
IL-6	sense	5'-CAGGAGAAGATTCCAAAGAT-3'	459 bp	[36]
	anti-sense	5'-ACTGGTTCTGTGCCTGCAGC-3'		
βactin	sense	5'-GTGGGGCGCCCAGGCACCA-3'	519 bp	[37]
	anti-sense	5'-TCCTTAATGTCAAGAACGAT-3'		

INTERNAL OLIGONUCLEOTIDE PROBES		T• HYBRIDIZATION	REFERENCE
GM-CSFR α	5'-TGTGACTCCTTCATGCAGAC-3'	55•C	[3]
GM-CSFR β	5'-GTGTGGTCTATGTGTTCGTA-3'	55•C	[5]
IL-3R α	5'-AAGAAATCCACGTCATGAAT-3'	50•C	[4]
IL-6R	5'-ATCCACAAACAACATTGCTG-3'	50•C	[7]
gp130	5'-GAATAATCAACAGTGCATGA-3'	50•C	[9]
GM-CSF	5'-ATTTCTGAGATGACTTCTAC-3'	55•C	[34]
IL-3	5'-CTCAAGGGTTTTCAGATAGA-3'	55•C	[35]
IL-6	5'-GTCTCCTCATTGAATCCAGA-3'	55•C	[36]
βactin	5'-AGGTCTCAAACATGATCTGG-3'	50•C	[37]

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TABLE 2 *Detection of cytokine receptor gene transcription by PCR amplification of reverse-transcribed total RNA derived from human malignant cell lines.*

	GM-CSFRα	GM-CSFRβ	IL-3Rα	IL-6R	gp130
HL-60	+	+	-	-	+
KGI	+	+	+	-	+
CESS	+	+	+	+	+
Small cell lung cancer					
H69	-	-	-	-	+
H128	-	-	-	-	+
OC1	-	-	-	-	+
OC2	-	-	-	-	+
OC3	-	-	-	-	+
FRA	-	-	-	-	+
STO	-	+	+	+	+
GIL	-	+	-	-	+
THO	+	-	-	-	+
BAT	-	-	-	-	+
DEV	-	-	-	-	+
VHE	+	-	-	-	+
FIC	-	-	-	-	+
DOZ	-	+	+	+	+
Melanoma					
DES	+	-	-	+	+
G43	-	-	-	-	+
WO	-	-	-	-	+
RENI	-	-	-	-	+
ADE	+	+	+	+	+
Head and neck cancer					
Fadu	+	-	+	+	+
KB	+	-	-	+	+
Hep-2	+	+	-	+	+
Detroit	+	+	+	+	+
RPMI 2650	-	-	-	-	+
Ovarian cancer					
AZ 224	+	-	-	-	+
AZ 382	+	+	+	-	+
AZ 364	-	-	-	-	+
AZ 303	+	-	+	-	+
Prostatic cancer					
DU145	+	-	+	+	+
Gastric cancer					
AGS	-	-	-	-	+
KATO III	+	-	-	+	+
Kidney carcinoma					
CAKI I	+	+	+	+	+
CAKI II	+	-	-	+	+
A498	-	-	-	+	+

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TABLE 3 *[^{3}H]-thymidine incorporation by selected malignant cell lines. 10^5 cells/ml were cultured in 0, 20, 100, 500 ng/ml of either human recombinant GM-CSF, IL-3 or IL-6. Incorporations shown represent mean cpm ± SEM of triplicate determinations following 6 days of culture. Concentrations of added growth factors are indicated to the right.*

CELL LINE	GM-CSF	IL-3	IL-6	
STO	1,855±453	1,219±19	1,515±152	0 ng/ml
	1,238±83	957±27	1,319±87	20
	1,689±242	1,656±103	1,788±194	100
	1,498±104	1,243±101	1,793±182	500
DOZ	95,929±1,181	97,556±2,693	113,510±764	0 ng/ml
	25,828±3,448	43,349±2,297	126,253±2,681	20
	69,057±1,174	95,911±2,405	118,358±3,228	100
	106,901±4,085	99,249±2,512	146,272±2,000	500
DES	75,070±2,044	87,981±13,540	100,406±7,922	0 ng/ml
	106,421±3,056	117,698±2,338	138,621±2,692	20
	113,433±4,797	110,762±1,483	107,408±2,203	100
	90,155±3,565	120,166±5,908	63,294±8,425	500
ADE	5,482±466	7,397±894	6,576±727	0 ng/ml
	8,978±1,374	9,162±104	9,652±1,419	20
	8,482±705	9,109±104	6,385±93	100
	6,624±407	7,494±176	9,133±555	500
Hep-2	16,774±431	17,888±255	18,089±3,225	0 ng/ml
	12,659±682	16,275±463	26,812±311	20
	13,141±108	18,457±581	29,779±352	100
	14,400±1,309	19,963±1,646	24,643±1,186	500
Detroit	30,368±1,221	42,428±8,135	45,904±4,589	0 ng/ml
	35,720±1,225	47,487±5,722	51,268±1,538	20
	39,203±6,238	57,645±6,118	56,484±7,263	100
	33,876±5,782	48,221±2,274	45,719±2,657	500

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TABLE 4 *Detection of cytokine gene transcription by PCR amplification of reverse-transcribed total RNA derived from human malignant cel lines.*

	GM-CSF	IL-3	IL-6
ST0	-	-	+
DOZ	-	-	+
DES	-	-	-
ADE	-	-	+
Fadu	-	-	+
Hep-2	-	-	+
Detroit 562	-	-	+
AZ382	-	-	+
DU145	-	-	+
KATOIII	-	-	-
CAKII	-	-	+
CAKIII	-	-	+
A498	-	-	+

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PROGRESS REPORT; JANUARY - DECEMBER, 1992

BIOLOGICAL BEHAVIOUR OF GENITAL HPV INFECTIONS

A Prospective Follow-up and Partner Study.

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&

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ABSTRACT

To assess the natural history of Human papillomavirus (HPV) infections in the lower female genital tract and their associations with intraepithelial neoplasia (CIN, VAIN, VIN) and genital squamous cell cancer, a long-term prospective follow-up study was started in October 1981. The project was the first of its kind, and still continues to be the largest (and oldest) ongoing prospective follow-up study in this field. The final number of women in the two Follow-up **Groups A and B**, is currently 530, including HPV-lesions with and without concomitant CIN (HPV-CIN, and HPV-NCIN lesions, respectively). The mean follow-up time of the whole series approaches 80 months by now. The recruitment of women into the third series, called the **Treatment Group**, currently comprising the same number (530) of patients as in Groups A & B, was discontinued in September, 1990, when the final size of the group was reached. During the current project period (**January-December 1992**), the recruitment of the male sexual partners of the Treatment Group women into the **Partner Group** has been continued. The number of the male partners enrolled has recently exceeded 300. **During the ongoing research period, the enrollment of a fifth series of patients was initiated, when small children and newborn babies born to mothers in the Follow-up and Treatment Groups are being recruited for clinical examination and sampling of their oral cavity and nasopharynx. The aim of this approach is to elucidate the possible non-sexual (i.e., vertical) transmission of HPV from the mother to her child and the biological significance of such an eventual transmission.** The follow-up of the women included in the two Follow-up Groups (A & B) as well as in the Treatment Group has been continued as done for over 10 years by now, using colposcopy, PAP smears and/or punch biopsy. The latter are examined by the techniques outlined in the Research Plan. These include histological assessment and grading as well as HPV typing by in situ hybridization and PCR. Cervical swabs were analysed for isolation of Chlamydia trachomatis and HSV, and serum samples for viral (HSV, CMV) serology. During the ongoing period, plans were made to initiate collaboration with German Cancer Reserch Centre to accomplish the serological analyses of the serum samples (thousands collected in the freezer between 1981-1992) for HPV type-specific antibody levels. The women in the Treatment Group are randomized according to four different treatment modalities; conization, laser therapy, cryotherapy, and interferon therapy. The HPV lesions of the male genitalia are examined by peniscopy, cytology, and biopsy (including HPV DNA typing and gene amplification by PCR), and treated by laser, whenever considered appropriate. The systematic HPV typing of the biopsies was continued using the in situ DNA hybridization (ISH) technique as a routine procedure. At the meantime, developmental work has been continued with the variety of DNA techniques to refine these methods applicable in routine laboratory praxis. As a result of this approach, a series of six commercial tests has been introduced into the market, which enable the HPV typing in both the biopsies and cell smears. During the current project period, biopsies have been tested for amplification of the cellular oncogenes (c-onc) by the ISH and PCR techniques. The role of p53 (a known antioncogene) has been assayed using immuno-histochemical staining in CIN lesions. As a new line of research, a series of samples has been analysed for mutations in p53 sequence by utilizing an automatic DNA sequencer. Binding of HPV E6 oncogen with p53 might have important implications in the malignant transformation by this virus. The complete HLA-typing of 100 follow-up women has been finally completed, and the results were submitted for publication. In the statistical treatment of the vast amount of data accumulated during these 10 years, a definite progress was made with the development of statistical models used to predict the disease outcome more accurately than possible at the moment. As a major part of the statistical analysis, the data derived from a case-control design (i.e., follow-up women and age-matched controls) were analysed to assess the factors increasing the risk for genital HPV infections. According to this analysis, the number of sexual partners during the past two years proved to be the single most important risk factor (RR >44 for women with 5 or more partners as compared to women with 1 partner only). The refinement of these data resulted in publication of yet another (MD) PhD Thesis (Dr. V. Kataja) in December. During the latter half of the period, a new technique was studied by Stina Syrjänen in DKFZ, Heidelberg (Prof. zur hausen); the organotypic culture (raft) of keratinocytes. This in vitro model will be established in our laboratory to be used in a number of different experiments, including the treatment trials with antisense oligonucleotides for HPV 16 E6/E7 mRNA.

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1.BRIEF SUMMARY

To assess the natural history of Human papillomavirus (HPV) infections in the lower female genital tract and their associations with intraepithelial neoplasia (CIN, VAIN, VIN) and genital squamous cell cancer, a long-term prospective follow-up was started in October 1981. The project was the first of its kind, and still is the most extensive (and of longest duration) ongoing prospective follow-up study in this field. The final number of women in the two Follow-up **Groups A and B**, is currently 530, including HPV-lesions with and without concomitant CIN (HPV-CIN, and HPV-NCIN lesions, respectively). The mean follow-up time of the whole series has exceeded 75 months by now. The recruitment of women into the third series, called the **Treatment Group**, currently comprising the same number (530) of patients as in Groups A & B, has been continued until September, 1990, when the final size of the group was reached. During the entire project period, the recruitment of the male sexual partners (of the Treatment Group women) into the **Partner Group** has been continued, their number currently reaching 265. The follow-up of the women included in the two Follow-up Groups A & B and in the Treatment Group was continued as done for 10 years by now, using colposcopy, PAP smears and/or punch biopsy. The latter are examined by the techniques outlined in the Research Plan (i.e., by light microscopy for HPV-induced changes and concomitant CIN, by immunohistochemistry for expression of HPV structural proteins, and immunocompetent cells using monoclonal antibodies to define B cells, T cell subsets, NK, natural killer cells and Langerhans cells). Cervical swabs were analysed for isolation of Chlamydia trachomatis and HSV, and serum samples for viral (HSV, CMV) serology. Blood samples have been also collected for analysis of T-cell subsets by flow cytometry (FACS). The patients in the Treatment Group were randomized according to four different treatment modalities; conization, laser therapy, cryotherapy, and interferon therapy with a cream. The HPV lesions of the male genitalia were examined by colposcopy, cytology, and biopsy (including HPV DNA typing), and treated by laser, whenever appropriate. The systematic HPV typing of the biopsies was continued using the in situ DNA hybridization technigue as a routine procedure. At the meantime, developmental work is being continued with the in situ hybridization and PCR (polymerase chain reaction) techniques, to refine these methods applicable in routine laboratory diagnosis. The complete HLA-typing of 100 follow-up women started earlier was concluded. In the statistical treatment of the vast amount of data accumulated during these 10 years, a progress was

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made with the development of the Prognostic Index, which could be used to predict the disease outcome more accurately than possible at the moment. Correlating the data obtained with the biological behaviour of the HPV lesions in these prospectively followed-up women, evidence on the factors regulating the clinical course of this increasingly common STD will be hopefully obtained. Tentative results are also available on immunohistochemical tests made to analyse the expression of a variety of HPV gene products (e.g., E6-encoded proteins) in the biopsies, using mono- and polyclonal antibodies generated against both synthetic peptides and fusion proteins. One of the future aims is directed towards the utilization of these data and such reagents in the development of serological tests for HPV infections. As a major part of the statistical analysis, the data derived from a case-control design (i.e., follow-up women and age-matched controls) were analysed to assess the factors increasing the risk for genital HPV infections. According to this analysis, the number of sexual partners during the past two years proved to be the single most important risk factor (RR >44 for women with 5 or more partners as compared to women with 1 partner only). Apart from the attempts to establish the prognostic factors for genital HPV infections, it will be of equal importance to outline the factors subjecting the people to clinical HPV infections which should be regarded as potentially premalignant conditions with significantly increased risk for genital cancer. Pertinent to this issue is another line of reserch initiated only recently; the exploration of the possible non-sexual transmission of HPV from an infected mother to her newborn baby. For that purpose, the babies and small children of the women in the follow-up are being examined for their HPV status in oral cavity and nasopharynx, by taking a swab to be analysed by PCR.

2.STUDIES CONDUCTED DURING THE BUDGET PERIOD (JANUARY-DECEMBER 1992)

Prospective Follow-up

During the budget period, the prospective follow-up of the women previously included in the series has been continued according to the original plans. The recruitment of new patients into the two Follow-up Groups A and B, has been terminated by the end of 1985; the final size of the cohort now being 530 women with HPV-infection (with or without concomitant CIN; HPV-CIN, and HPV-NCIN lesions, respectively). The mean follow-up time of the whole series approaches 80 months by now. During the ongoing period, a decision was made together with the clinical collaborators, that the

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follow-up of Groups A and B will be terminated during 1993-1994. This will be done as a highly organized manner by completing an end-point examination with extensive sampling and councelling for the future control of each individual woman. By that procedure and schedule, a mean follow-up time for the whole series becomes close to 10 years, which will be enough to elucidate the natural history of genital HPV infections.

Treatment

Starting from 1985, the third series of women has been recruited for this prospective follow-up study. This series is designated as the Treatment Group, currentiy comprising 530 women with HPV-CIN lesions. These patients are randomized into four distinct groups, each being treated with different modalities; conization, CO_2 laser vaporization, cryotherapy, and interferon treatment (topical administration as a cream). The close post-treatment follow-up of these women will disclose the efficacy of the above therapeutic measures on the clinical course of HPV infections. Most importantly, however, the study design permits the direct comparison between the natural history of this disease (i.e., spontaneous regression rate) and the clinical cure achieved with the active treatment. The follow-up of the Treatment Group women will be continued at least until the mean follow-up time will reach 5 years, i.e. until 1995-1996.

Examination of Samples

The women in all three series (A & B, Treatment Group) still attend the clinic at 6-month intervals, and on each attendance they are subjected to colposcopy, accompanied either by a directed punch biopsy and/or PAP smears. These are examined, as previously indicated, for the presence of HPV-induced cytopathic changes, concomitant CIN, HPV DNA by in situ hybridization and gene amplification by PCR. The role of accompanying cervical infections (possibly showing synergistic actions with HPV) is assessed by cervical swabs (for isolation of Chlamydia and HSV), and by serum samples for viral (HSV, CMV) serology. More recently, specific DNA probes and primers have become available which enable the direct detection of these agents in the biopsies or cellular swabs by ISH and PCR, respectively. In fact, our group has been involved in developing some of these tests into diagnostic use (see below).

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The fresh biopsy samples have been collected from all patients (at each attendance) and stored at -70°C for future analysis. So far, these have been used for Southern blot hybridization and for immunohistochemical staining of the immunocompetent cells (on frozen sections) within the local inflammatory infiltrate. Using monoclonal antibodies (McAb), the following cell types have been defined; B cells, T cell subsets, NK (natural killer) cells and Langerhans cells. These cells are currently being analysed also in the peripheral blood, using monoclonal antibodies and and flow cytometry by FACS. These data might aid elucidating some of the evidently complex immunological reactions involved in biology of HPV infections, recently accentuated by the attempts towards development of vaccines against papillomaviruses.

Oral Cavity as HPV Reservoir

During the ongoing research period, Dr. Jari Kellokoski, DDS, completed his PhD. Thesis which is the the first systematic approach to assess the biological significance of HPV infections in the oral cavity. The specific aims and results of the study were as follows: **1)** To classify the morphological changes in the biopsies from buccal mucosa of women with genital HPV infection, and to evaluate the usefulness of oral scrapings, as well as morphological and histological criteria in assessing HPV infections in oral mucosa. **Results: By clinical inspection, the HPV association of tiny lesions is difficult to predict. On light microscopy, vacuolized cells were frequently encountered in clinically normal buccal mucosa. No correlation between vacuolized cells and koilocytes to HPV DNA content could be established. 2)** To analyse whether acetic acid staining can be applied on the specific diagnosis of oral HPV infections, and to further elucidate the factors ralated to the acetowhite staining, icluding the **smoking and drinking habits** and cytokeratin patterns in the epithelium. **Results: Acetic acid staining proved to be of no value in detection of HPV infection on oral mucosa. Instead, it was associated with smoking and seemed to be inversely age-dependent. In the biopsies containing HPV DNA, cytokeratin no.19 staining was intensified in the basal layer. 3)** To determine the sensitivity of differnet hybridization methods in detection of HPV infection and to oral HPV infections in buccal mucosa in women with genital HPV infections. Results: **Dot blot hybridization detected HPV DNA in 3.8% of the cases, while 21.8% proved to be positive with PCR. Dot blot positivity reflects only clinical and subclinical HPV infections. With Southern blot hybridization,**

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15.1% of biopsies taken from clinically normal buccal mucosa contained HPV DNA. 4) To assess HPV transmission by correlating the HPV types in the oral mucosa of the patients and those on the anogenital mucosa of their partners. **Results: Only a single patient had the same HPV type both in the genital and oral mucosa. Preliminary data on the anogenital HPV types in the male partners showed that none of them had the same HPV type as their female partners in the mouth, which suggested that transmission of HPV by oral-genital sex was unlikely.**

Non-Sexual Transmission of HPV

The above studies raised several questions which are the subject of the present study as follows: **1)** To explore the non-sexual routes of HPV transmission by examining the oropharyngeal swabs of newborn babies born to mothers with clinical HPV infections of the uterine cervix. **2)** To assess the clinical significance of such an eventual transmission of HPV (whether a true infection or a passenger) by sequential swabs of infants born to mothers with clinical HPV infections of the cervix. **3)** To analyse the clinical course of oral HPV infection in the infants. **4)** To establish the prevalence of HPV infections (e.g. skin warts, oral HPV infections, whether latent, subclinical or clinical) in children born to mothers with known genital HPV history. **5)** To correlate the HPV types in children to those of their parents.

The subjects to be studied are volunteer children born to women in the follow-up series since 1981. All children (appr. 250) are invited to attend the clinical examination for their oral HPV infections. The examination includes a clinical inspection, collection of saliva as well as oral scrapings to be analysed by PCR to detect HPV sequences. All clinical lesions will be biopsied. If the child is HPV-positive, then the virus typing data will be correlated to the HPV status of the parents. HPV-positive children will be followed-up for several years with regard to their oral mucosal lesions. The second study group consists of newborn babies (approx. 30) born to mothers with clinical HPV infection of the cervix. As control groups, a series of babies (about 50) born to healthy (i.e., non-HPV-infected) mothers, and 30 babies delivered by cesarean section will be included in the study. From all babies, a nasopharyngeal swap collected by suction immediately after delivery will be analysed by PCR to detect HPV DNA. All babies will be repeatedly analysed on the 4th day. The HPV DNA-positive babies will also be examined at the age of 6 weeks, 6 months and one year. The infants will be followed by oral scrapings. Pap

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smears will be taken from the mothers just before the delivery. This is the only study design, by which the eventual vertical transmission of HPV can be assessed, and the question whether HPV is a passenger or a true infective agent can be reliably answered.

Male Sexual Partners

The male sexual partners (Partner Group) of the women included in the Treatment Group are being recruited for examination, treatment and follow-up. Since the start of this recruitment in late 1986, the genital HPV lesions of >300 males have been examined by peniscopy (after acetic acid application), cytology, and biopsy including HPV DNA typing. Also the male HPV lesions are treated by laser, whenever appropriate. According to the data elaborated by Dr. Hippeläinen, the role of peniscopy as an important diagnostic tool in penile HPV lesions has been emphasized. It is important to realise, however, that peniscopy is not the conclusive diagnostic tool, because of its limited resolution, rendering it incapable of distinguishing between HPV and non-HPV lesions.

From the epidemiological standpoint, the concordance of HPV types between the sexual couples has become of prime importance. This concordance of a) genital HPV lesions and b) viral types in HPV-infected women and their male sexual partners, was analysed by Dr. Hippeläinen in her series of 282 women and their male partners examined by peniscopy and punch biopsy. Of the males, 199 (70.6%) showed histological evidence for HPV infection, 89.4% of which being flat lesions. HPV DNA was found in 181 (38.0%) of the 476 biopsies examined by ISH, exophytic warts (26 cases) and PIN lesions (20 cases) being most frequently HPV DNA-positive (92.3% and 80%, respectively). HPV DNA was also detected in 76 (31.3%) flat lesions histologically not equivocal HPV, i.e., in subclinical infections. Most importantly, of the 271 sexual couples subjected to HPV typing, both partners were ISH positive in 66 (24.4%) cases, but **only 15 (5.5%) couples had an identical HPV type** in their genital tract. The implications of these findings remain to be elucidated in the future studies.

One of the major aims of the partner study is to assess the effects of treatment given to the males on the clinical course of HPV-infections in their female sexual partners (included in the prospective follow-up series). The elucidation of this issue will also have major relevance in the proper evaluation

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op the treatment policy, and eventually might result in tremendous save of expenses, if the current hypothesis on the minor benefit achieved by the male treatment proves to be correct.

Asymptomatic Males

As a part of the male studies, a novel approach was made during the present study period, to determine (i) the prevalence of and (ii) risk factors for genital HPV infections in healthy males. Voluntary Finnish Army conscripts were examined using peniscopy after acetic acid, surgical biopsy, cytology and PCR for detection of HPV DNA in brush cytology samples collected from the urethra and the penile epithelium. The HPV data were complemented with a questionnaire inquiring their sexual habits. A total of 1471 (99.6%) males filled the questionnaire, and 432 of them attended the clinical study. The group entered in the examination differed from the nonattenders in that they reported more often genitourinary symptoms ($p=0.000$), had more sexual partners ($p=0.002$), and a previous history of sexually transmitted diseases (STDs) ($p=0.000$). Classical genital warts were present in 24 (5.6%) and papular lesions in 8 (1.9%) of the 432 men. Acetowhite epithelium was disclosed in 151/432 (35.0%) cases, of whom only 75/432 (17.4%) had obvious flat HPV lesions on peniscopy. Histological diagnosis of HPV infection was confirmed in 28 of the 42 (66.7%) biopsies taken from different types of lesions. HPV DNA was disclosed by PCR in 16.5% (47/285) of the adequate cell samples. Altogether, 26.2% (113/432) of the subjects were HPV-positive by at least one of the diagnostic methods, whereas only 6.5% (25/383) had a diagnostic peniscopic pattern and positive PCR. Furthermore, HPV DNA was found in 7.1% of the men with normal epithelium. In the logistic regression analysis, occasional sexual partners, previous STDs and no use of condom proved to be of predictive value for current HPV infection.

Prevalence and Incidence

The collection of the data on risk factors with suggested influence on transmission of the HPV infections has been continued by the detailed questionnaire for the sexual and smoking habits of the patients. Meantime, the annual mass-screening programme for cervical precancer lesions run by the Finnish Cancer Society Laboratory of Pathology in Kuopio has been utilized to define the prevalence of clinically manifest (i.e. detectable by PAP smear) genital HPV infections in unselected population of

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25-60 year-old women. The prevalence figures of 1991 were elaborated in early 1992, and the declining trend in disease prevalence noticed since the top year 1987 (1.77%) seems to continue, i.e. the figures falling down to 0.77% in 1991.

Risk Factors and Prognosis

The life-table method and log-rank analysis by Peto were used to assess the differences in clinical course between the groups of HPV lesions classified according to different variables; histologic grade, PAP smear grade, HPV type, colposcopic pattern etc. The survival tables were produced and the survical curves plotted with SPSS^{x} computer programme utilizing the product-limit (Kaplan-Meier) method. Estimation of reproducibility in diagnostic histopathology is also possible by applying the kappa statistics. This statistics takes into account the contribution of chance agreements. The intraobserver reproducibility and variation have been calculated for assessment of the punch biopsies and PAP smears.

The epidemiologist in our group, Dr. Vesa Kataja completed his doctoral Thesis in December. Im his work, the statistical techniques play a central role. The main purposes of his epidemiologic study were *a)* to establish the prevalence of cervical HPV infections in mass-screened Finnish female population aged 20-65 years, *b)* find out the incidence of the infection in a high-risk age group of 22-year old women, *c)* to elucidate the risk factors associated with clinical HPV infections, *d)* to assess the natural history of the disease during a long term prospective follow-up, and *e)* to establish prognostic factors predicting the disease outcome. The prevalence of cervical HPV infections was studied using cervical Pap smear in a conventional mass-screening in Kuopio County. The prevalence increased from 0.04% in 1981 to 1.76% in 1987, thereafter decreasing to 1.43% and 1.04% in two subsequent years. The prevalence varied significantly with age. The highest prevalence, 6.1%, was seen in the age group 20-29 years. In an extra mass-screening specially designed for a cohort of 22-year old women, the prevalence was studied in two successive years, 1985 and 1986. The prevalence in the first year was 2.7% and in the second year 7.1%, resulting in a crude annual incidence of 7.0% in this age group. Based on these figures, 79% of the Finnish female population would contract at least one genital HPV infection between the ages 20-79.

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The strongest independent risk factor for clinical HPV infection proved to be the increasing number of sexual partners within the past two years. Current smoking, previous CIN, STD and condyloma acuminatum were other factors associated with an increased risk for HPV infection. Regular use of IUD and good personal hygiene practices were among the factors with protective effect on the infection. During the mean follow-up of 79.2 months, 63.8% of the genital HPV infections (in 530 women) have spontaneously regressed without any treatment. Progression has occured in 13.8% of the cases. The strongest independent prognostic factors for progression were the high grade of CIN and HPV type 16. The adjusted relative risks for CIN I, CIN II and CIN III were 1.6, 2.9, and 9.6, respectively. The study provides basic epidemiologic data on genital HPV infections, which are the most prevalent STD of today. These data are also essential in defining the strategies for the management of these women.

Organotypic Culture as In Vitro Model

A significant impediment of HPV study has been the lack of a cell culture system that allows for expression of the complete viral life cycle. A cell culture system permissive for vegetative viral replication would be valuable not only to the understanding of PVs but also for the treatment and prevention of HPV-induced lesions. HPV life cycle has been shown to be coupled with the differentiation programme of keratinocytes, and a major obstacle to the propagation of HPV *in vitro* has been the inability to mimic the proliferation/differentiation cycles of HPV target tissue, the squamous epithelium. The organotypic culture system (raft) mimics the *in vivo* physiology of the epidermis by raising the cells to the air-liquid interface. This can be done either by the recombination of epidermal cells with dermal elements or through the use of a collagen matrix maintained on rigid support. Growing keratinocytes in either of these systems allows for a more complete differentiation programme to occur than is observed in monolayer.

To date, a number of modifications on the raft system exists. The basic technique used by most laboratories was introduced in 1984. In brief, fibroblasts are mixed with medium and collagen I at 0•C to 4•C and the placed into tissue culture dishes and incubated at 37•C until the solution solidifies. The collagen fibers and the fibroblasts form a lattice (plug) which is used as the dermal equivalent. Epidermal cells are seeded on top of this dermal equivalent, allowed to attach and form a monolayer.

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The plug is then lifted on to a metal grid, and fed so that the media comes only in contact with the bottom of the grid, maintaining the epidermal cells at the air-liquid interface. During the period of two to three weeks, the epidermal cells stratify and differentiate. The collagen plugs are then removed from the metal grids and prepared by standard techniques for histological examination.

The raft model has recently gained wide applications in HPV research. The utility of this system in our project was recognized recently, and facilities were offered to learn the technique in Prof. zur Hausen's laboratory in German Cancer Research Center, Heidelberg. A two-week visit there was done in early December by Dr. Stina Syrjänen, who is currently establishing the system in our laboratory.

Antisense Oligonucleotides in Experimental Therapy

Recent studies have focused on the ability of oligonucleotides to affect the genetic processes on many organisms, incuding viruses. Hence, oligonucleotides may represent a future source of biotechnologically derived compounds of therapeutic importance for several diseases such as cancer and viral infections. The viruses function through the transfer of genetic information from DNA to mRNA, and further to proteins. In case of retroviruses, the information originates from the viral RNA. These genetic processes can be intervented using oligonucleotides. The most widely used approach to interrupt the flow of genetic information is to use oligonucleotides as inhibitors of translation, with the complementary or "antisense" base sequence targeted to a specific "sense" sequence in the mRNA. Thus, expression of specific proteins can be regulated or inhibited. The preferred target for this antisense inhibition is the 5'-initiation codon. In general, an oligonucleotide of 15-20 bases is used for the antisense inhibition. The in vitro applications being successful so far include inhibition of expression of several oncogenes (c-myc, N-myc, myb) and HIV.

We have recently started a novel approach, where the ability of antisense oligonucleotides to inhibit the expression of HPV 16 and HPV 18 oncoproteins E6 and E7 18 is assessed in vitro. We have selected several antisense oligonucleotides to interact with target the mRNA of HPV 16 and HPV 18 in CaSki and HeLa cells, respectively. These oligos are automatically sulphurated (phosphorothiolate linkages) during the synthesis. The phosphorothiolate linkage makes the antisense oligos more

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resistent to nuclease degradation. We are using cationic lipids that form liposomes to facilitate the functional delivery of oligos into cells. Both the quantity of DNA and concentration of lipid required for optimal signals are being tested. According to our preliminary results, optimal conditions depend on the cell types used and differences exist in the reaction conditions between CaSki and HeLa cell lines. The interaction of liposomes with the DNA and the delivery of the antisense oligonucleotides into the cell are controlled by confocal laser scanning microscope (CLSM) by labelling the liposomes with rhodamin and the oligos with FITC. The effect of inhibition is assessed by analysing the expression levels of E6 and E7 mRNAs by Nothern blotting and quantitative RNA PCR. Extensive in vitro testing is a prerequicite for in vivo applications to HPV-infected patients.

Role of Anti-Oncogenes p53 and RB

The best known genes of this group are the retinoblastoma susceptibility gene (RB) and p53 gene. Both the RB protein and the p53 protein are interesting in that they are able of forming stable complexes with transforming proteins of several DNA tumor viruses. This would indicate the possibility that cellular tumor suppressor gene products may be inactivated by binding to viral oncoproteins. It has been shown that the E7 protein of HPV also has the ability of binding the RB protein and that the E7 protein shows sequences homologies to parts of the adenovirus EIA protein and the SV40 large T antigen involved in RB binding. Although the E7 protein of all HPVs can bind to RB, the E7 protein of HPV types associated with carcinomas, e.g. HPV 16 and 18, shows a higher affinity than the E7 gene product of HPV types found in benign lesions. It has also been demonstrated recently that HPV 16 E6 protein binds p53 tumor suppressor gene product, which usually acts as a negative regulator of cell proliferation.

The expression of tumor suppressor genes is changed in several tumors due to genetic alterations like deletions and mutations. In tumors the expression of mutant p53 is frequently increased when compared to wild-type p53 in normal cells. Due to the longer half-time of p53 expression as detected by immunohistochemistry is usually interpreted as the expression of mutant p53, not as the wild-type p53 expression present in cell cycle G_0-G_1 phase in proliferating cells. HPV 16 and 18 E6 protein binding to wild-type p53 has been found to enhance the degratation of the product by the ubiquitin-

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dependent protease pathway. The potency to form a complex with p53 diverges from a HPV-type to another. While the E6 proteins from HPV 16 and 18 bind to p53, the E6 proteins from HPV 6 and 11 serotypes fail to detectably bind with p53. This is of some interest because HPV 16 and 18 have been mostly found in cancers, therefore were called as 'high-risk' HPV types; while HPV 6 or 11 are most frequently associated benign condylomatous lesions.

Functional inactivation of wild-type p53, caused by either genomic mutations or neutralization by the transforming proteins from several tumor viruses, is currently regarded as an important molecular pathway for the pathogenesis of human malignancies. Theoretically, if HPV infections do play an etiological role in the pathogenesis of anogenital carcinomas, then one can easily estimate that cervical carcinomas associated with HPV should contain only a wild-type p53 gene (which would be neutralized and inactivated by binding to E6 protein), whereas cervical carcinomas without HPV would have mutant p53 genes (i.e. mutational inactivation of wild-type p53). This is indeed the case. Preliminary studies of cervical carcinomas and carcinoma derived cell lines have revealed point mutations within the p53 sequences only in HPV negative tumors. Thus, it seems that the inactivation of tumor suppressor genes may be a common mechanism for the transformation of cells shared by several DNA tumor viruses, including HPVs.

In our project, we have started to investigate the expression and mutations of p53 gene in dysplastic lesions and carcinomas immunohistochemically using PAb1801 antibody, which recognizes both the wild-type and mutant p53, as well as by p53 DNA sequencing with PCR amplification. Presence of HPV infection in these lesions will be demonstrated by PCR using HPV concensus (Manos et al 1990) and HPV 16 and HPV 18 type-specific primers. Amplified DNAs from PCR using concensus primers are blotted from agarose-gel onto filter and hybridized with biotinylated HPV-probe in low stringency to confirm the presence of HPV-DNA. Meanwhile, selected p53 DNA sequences in the lesions will be amplified by using specific primers chosen from the exon 5-9 (which are known as the most frequent area of point mutations) of the p53 genes. The amplified PCR products will be cloned into plasmid and subsequently sequenced using the chain-termination method, and the DNA sequences are determined by the automated DNA sequencer which is available at the Department of Biochemistry &

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Biotechnology. Expression level and gene sequence data of p53 will be compared with the presence of HPV-DNA. Similar techniques are being used to assess the role of RB gene expression and mutation. It will be of utmost interest to evaluate whether the detection or changes in these two anti-oncogenes are of any prognostic value in genital HPV lesions.

Development of Diagnostic DNA Tests

During the ongoing budget period, critical evaluations have been continued on the sensitivity and applicability in clinical use of the different DNA-hybridization techniques; Dot blot, Southern blot, in situ, as well as the role of PCR in diagnosis of HPV. These techniques have been extensively used in detecting and typing of HPV DNA in the clinical samples so far. To supplement the diagnostic pattern previously including the HPV DNA probes for HPV type 6, 11, 16 and 18, additional probes of HPV types have been acquired. At the moment, the **DNA probes of all known HPV types** are at our disposal. A systematic analysis (using ISH with seven different probes, HPV 6,11,16,18,31,33,42) of all the biopsies collected since 1981 was started in 1986. This technique is currently used in our laboratory as a routine method of typing the biopsies derived from the above four series of patients, and data are available of more than 4.000 biopsies by now.

As a part of our research programme, continuous developmental work has been done (as a joint effort with a Finnish biotechnology company, BIOHIT) for several years by now. A major goal of this joint venture has been to develop the laborous and complicated DNA hybridization techniques more applicable to routine pathology laboratory. During the year 1990, this line of research led into the first commercial products, when an in situ hybridization kit based on biotin-labelled whole genome HPV DNA probes was introduced to the international market (BioHit^{R}, HPV in Situ Typing Kit). This has subsequently led into the entire family of related products, including a sampling kit, a screening kit, as well as a selection of HPV-positive sample slides. This collaboration has been progressing also during this year, when the previously introduced kits for EBV (BioHit^{R} EBV in situ Test Kit), and the first non-radioactive dot blot test to screen and type HPV infections (BioHit^{H} HPV dot blot Screening Test and -Typing Test) were further improved. A new set of diagnostic tests is to be expected in the near future. These "second generation" HPV diagnostic reagents will be based on the use of synthetic

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oligonucleotide probes. The generation of such reagents in our laboratory became possible in June 1990, when a DNA synthesizer (Gene Assembler Plus, Pharmacia) was obtained in the premises. The technique to prepare the biotin-labelled oligonucleotide probes ready for ISH was recently developed in our laboratory (S.Syrjänen et al., J.Med.Virol. 1990).

Role of PCR

The gene amplification by PCR technique was introduced to our laboratory in early 1989, when the appropriate automatic PCR machine was acquired (Perkin Elmer Cetus, Thermal Cycler). PCR is currently recognized as the most sensitive technique of all, capable of disclosing less than a single copy of HPV DNA per cell. At the same time, however, there is a significant risk of false positive results due to DNA contamination (from a variety of sources). The successful application of the PCR technique also critically depends on the selection of correct primers. Because of the fact that most groups have used the primers designed by their own, considerable variation and discrepant results have been published of the detection rate of HPV by different laboratories. Accordingly, HPV DNA has been reported in from 2-3% to >80% of genital biopsies derived from 'normal epithelium'. According to our own experience, some 30% of the biopsies derived from the lower genital tract mucosa of the women with a normal recent PAP smear were shown to contain HPV DNA when subjected to PCR analysis (for HPV 6,11,16 and 18). Results in line with these have been also obtained while analysing the genital biopsies of males, where the HPV DNA detection rate by using PCR remains close to 30% even if their sexual partners have established HPV lesions. Beyond any doubt, then, much of the recent PCR data must be basically incorrect. Thus, it is clear that a substantial amount of standardization work in the technical aspects of the PCR method is necessary before the elucidation of its role in HPV diagnosis is possible. Such a work is continuously being done in our laboratory as well. As a recent progress in this field, an approach was developed in our laboratory, by which an accurate quantitation of the PCR amplification is possible.

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Current Concepts and Future Outlook

A number of original reports, review articles and book chapters based on the results of the ongoing project have been published during the budget period (See the List of Reports). The results have also

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been presented in a number of international scientific congresses, as well as by giving invited guest lectures in a number of meetings abroad. Along with the extension of the prospective follow-up, our preliminary observations have been further confirmed suggesting that a certain proportion (about 15%) of the clinically manifest genital HPV infections, if left untreated, are capable of making definite progression, eventually into carcinoma in situ (CIS). These lesions thus present with the natural history identical with that of CIN lesions as established by well controlled prospective follow-up studies long before the association with HPV was recognized.

On the basis of the current understanding of the epidemiology of HPV infections, it would be of paramount clinical significance to establish the means to accurately predict the clinical course of HPV infections. Such a prediction should include the recognition of high risk lesions (i.e., those likely to make a rapid progression) from the low risk lesions liable to undergo a spontaneous regression without any treatment. To accomplish this, an extensive prospective study is mandatory. Our programme has provided some important data on this issue as well. During the 11-year follow-up (1981-1992), it has become apparent that the natural history of genital HPV-infections can be predicted (to certain extent) by the grade of the primary lesion at diagnosis. Accordingly, HPV lesions without CIN are more likely to regress sponatenously than those with CIN (HPV-CIN lesions). The latter, in turn, possess a high risk for progression as analysed by the life-table method. Along with the extension of the follow-up period, the dramatic difference originally suggested in the clinical behavior between the lesions infected by the low risk (HPV 6 and 11) and high risk (HPV 16,18,31,33 etc.) types has turned out to be less dramatic, albeit the highest risk for clinical progression seems to be associated with HPV 16 infections. In fact, the risk of progression for HPV 16 lesions seems to be more than 5-fold as compared with that of HPV 6- or HPV DNA-negative lesions. HPV type, on the other hand, could not be used to accurately predict the lesions with eventual spontaneous regression, albeit the regression rate of HPV 16 lesions seems to be very low indeed. At present, too little data are availabe on the biological behavior of the infections by the more recently described HPV Types 31, 33, 35, 39 and 42, and of the 50- and 60-series, some of which have been shown to be associated with the high grade CIN lesions and cervical cancer. **To fully elucidate the prognostication of HPV infections, more data are needed about the factors involved in regulation of the viral life cycle within the host cells,**

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including the growth factors, their receptors, as well as the complex interplay between the divergent hormonal factors, oncogenes, anti-ongcogenes etc. Once again, the full understanding of these mechanisms **necessitates a long-term prospective study** with extensive patient material. Such a set-up is available in Kuopio, where hopefully this follow-up project can be continued for a number of years ahead.

3.RESEARCH INVOLVING HUMAN SUBJECTS

The study protocols performed in the past year for human subjects in Group A and B have been explained in detail in the grant application. Because these protocols have remained the same, they will be explained only shortly in this context. As detailed for the subjects in Group A and B, the HPV-positive PAP smear is the single and only criteria of their inclusion in the study. The same is true with the recruitment of the women into the Treatment Group. Only the women presented with unequivocally HPV-positive smears containing changes consistent with CIN are invited in the Treatment Group. This practice is in full agreement with the general policy in Finland, according to which all women with cytological changes suggesting CIN should be referred to a colposcopic examination, independent of whether HPV infection is present or not.

The recruitment of the subjects into the three study groups was accomplished by an invitation letter, mailed along with the pathologist's report of the PAP smear. Thus, it is completely up to the patient to decide if she wants to enter the study and also to terminate the follow-up at any stage of the study. The detailed characterization of the specimens as well as the means of obtaining them have been given in the grant application, and these procedures in the Treatment Group do not differ in any way from those of Group A and B. Thus, the collection of the research and diagnostic material in the present study is accomplished by utilizing well established techniques in worldwide diagnostic use to monitor the genital infections and precancerous lesions.

The treatment modalities instituted to the women in the Treatment Group only include the established treatment techniques; conization, CO_2-laser vaporization therapy, cryotherapy, as well as interferon (as a topical cream or s.c.injections), the efficacy of which is currently being explored in many laboratories.

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As in any diagnostic and therapeutic clinical work, certain minor risks exist, which are unpredictable. During the 10-year existence of this project, however, no major complications have developed in any of the patients. It is completely clear, then, that the patients in the present three series are not subjected to any risk major than due to the diagnostic procedures used (colposcopy, PAP smear, punch biopsy, cervical swab, serum sampling, interview anonymously by a questionnaire), as well as due to the therapeutic procedures (conization, laser, cryo, and interferon). No alternative procedures are available to conduct an adequate prospective follow-up of genital HPV lesions (either non-treated or treated) other than those used in the present study, and repeated at intervals regarded safe to ensure that none of the HPV lesions will escape an early detection and proper treatment, whenever making progress.

The above diagnostic and therapeutic protocols also apply to the male sexual partners included in the Partner Group. This is also true with their continuous monitoring by the gynecologists responsible for their examination in the Outpatient Department of Gynecology and Obstetrics, Kuopio University Hospital. The above diagnostic protocols apply equally to the newborn babies and children enrolled for oral examinations since June 1992, after being accepted by the Ethical Committe of Kuopio University.

As previously emphasized, the risks to which the present patients are subjected are limited to those due to the application of the commonly accepted diagnostic and therapeutic procedures. The anticipated benefit of the patients while included in this most carefully controlled prospective follow-up study significantly exeeds the neglible risks to be expected from the use of colposcopy, PAP smears, punch biopsies, cervical swabs and serum sampling as well as conization, laser, cryo or interferon cream treatment. This benefit will be even accentuated, when the follow-up results are compared with the clinical outcome of the HPV lesions in non-controlled or even in mass-screened female population, subjected to PAP smears irregularly or at 5-year intervals, respectively. This is self-evident also for the males, now being examined and treated, but who, outside this study usually present with subclinical lesions indiscernible without colposcopy, and as such form a potential reservoir of HPV infections to be transmitted to their sexual partners.

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4.PROJECT BUDGET, January - December 1992

Items	Costs (FIM)
Technical Assistants (Full time, 2 x 12 months): Mrs. Helena Kemiläinen (1.1.-31.12.92) Mrs. Kaarina Hoffren (1.1.-31.12.92)	275.000,-
Computer Operator, (Full time, 1 x 12 months): Ms. Paula Lipponen (1.1.-31.12.92)	125.000.-
Material costs: Laboratory chemicals, reagents, isotopes, immunohistochemical kits, DNA reagents etc.	318.350.-
Printing costs: Reprints, page charges, colour prints	22.500.-
Congress costs: Travel expenses, congress fees, accomodation	10.500,-
Total Costs	751.350.- (FIM)
(USD 80.000,- + USD 95.000,-)	175.000.- (USD)

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4.REPORTS PUBLISHED DURING THE BUDGET PERIOD (JANUARY-DECEMBER 1992)

A) Original Reports

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2.Yliskoski, M., Saarikoski, S. and Syrjänen, K. Conization for CIN associated with Human papillomavirus infection. Arch. Gynecol. Obstet. 249, 59-65, 1991.

3.Yliskoski, M., Tervahauta, A., Saarikoski, S., Mäntyjärvi, R. and Syrjänen, K. Clinical course of cervical Human papillomavirus lesions in relation to coexistent cervical infections. Sex. Transm. Dis. 19, 137-139, 1992.

4.Kataja, V., Syrjänen, K., Syrjänen, S., Mäntyjärvi, R., Yliskoski, M., Saarikoski, S. and Salonen, J.T. Prognostic factors in cervical human papillomavirus (HPV) infections. Sex. Transm. Dis. 19, 154-160, 1992.

5.Syrjänen, S., Andersson, B., Juntunen, L., Syrjänen, K.J. Polymerase chain reaction for producing biotinylated Human papillomavirus DNA probes for in situ hybridization. Sex. Transm. Dis. 19, 140-145, 1992.

6.Kellokoski, J., Syrjänen, S., Yliskoski, M. and Syrjänen, K. Dot blot hybridization in detection of human papillomavirus (HPV) infections in the oral cavity of women with genital HPV infections. Oral Microbiol. Immunol. 7, 19-23, 1992.

7.Syrjänen, K. Human papillomavirus infection of the female genital tract. Editorial. Cytopathol. 2, 225-227, 1991.

8.Syrjänen, K.J. Genital human papilloma virus infections in HIV-infected males and females. In: Melica F (ed). AIDS and Human Reproduction, Karger, Basel, pp. 74-83, 1992.

9.Kataja, V., Syrjänen, S., Yliskoski, M., Hippeläinen M., Väyrynen, M., Saarikoski, S., Mäntyjärvi, R., Jokela, V., Salonen, JT, and Syrjänen, K. Risk factors associated with cervical human papillomavirus (HPV) infections: A case-control study. Am. J. Epidemiol. Submitted and Revised.

10.Yliskoski, M., Saarikoski, S., Syrjänen, S. and Syrjänen, K. Cryotherapy, CO_2-laser vaporization and conization in the treatment of cervical human papillomavirus (HPV) infections of known viral types. Obstet. Gynecol. Submitted.

11.Syrjänen, K.J. Histology of genital papillomavirus infection. Proceedings of the 7th World Concress of Cervical Pathology and Colposcopy, Rome, May 13-17, 1990. In press.

12.Tuppurainen, K., Raninen, A., Kosunen, O., Kankkunen, J-P., Kellokoski, J., Syrjänen, S., Mäntyjärvi, M. and Syrjänen, K. Squamous cell carcinoma of the conjunctiva. Failure to demonstrate HPV DNA by in situ hybridization and polymerase chain reaction. Acta Ophthalmol. 70, 248-254, 1992.

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15.Tosi, P., Cintorino, M., Santopietro, R., Lio, R., Barbini, P., Ji, H., Chang, F., Kataja, V., Syrjänen, S. and Syrjänen, K. Prognostic factors in invasive cervical carcinomas associated with human papillomavirus (HPV): Quantitative data and cytokeratin expression. Pathol. Res. Pract. 188, 866-873, 1992.

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21.Syrjänen, K.J. Papilloma virus and cervical cancer. Vox 14, 3-12, 1992.

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23.Costa, S., Syrjänen, S., Vendra, C., Chang, F., Guida, G., Tervahauta, A., Hippeläinen, M. and Syrjänen, K. Detection of Human papillomavirus infections in the male sexual partners of women attending an STD clinic in Bologna. Int. J. STD & AIDS. 3, 338-346, 1992.

24.Syrjänen, K.J. Does Human papillomavirus (HPV) cause cervical cancer? Int. J. Clin. Virol. 1, 2-6, 1992.

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26.Syrjänen, K.J. The normal cervix. Concept of the transformation zone. Proceeding of the 1st Europaen Course on genital HPV-associated pathology. Cervix & l.f.g.t. 10, 83-88, 1992.

27.Syrjänen, K.J. Cytology and histology of cervical lesions. Proceeding of the 1st Europaen Course on genital HPV-associated pathology. Cervix & l.f.g.t. 10, 89-94, 1992.

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29.Syrjänen, K. The long-term consequences of genital HPV infections in Women. Editorial. Ann. Med. 24, 233-235, 1992.

30.Syrjänen, K.J. HPV typing: Does it help or not? Proceedings: Nordisk Förening för Ostetrik och Gynekologi, XXVIII Kongress. Reykjavik, Iceland, June 9-12, 1992. Submitted.

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B) Abstracts

1.Syrjänen, K. Viral infections and genital cancer. The VIIth Meeting of the Scandinavian Society for Genitourinary Medicine (SSGU), Trondheim, Normay, February 13-16, 1992.

2.Syrjänen, S., Kellokoski, J., Yliskoski, M., Syrjänen, K. Oral HPV infections in women with genital HPV lesions. Evaluation of the clinical, morphological and HPV DNA findings. The VIIth Meeting of the Scandinavian Society for Genitourinary Medicine (SSGU), Trondheim, Normay, February 13-16, 1992.

3.Syrjänen, K. Papilloomavirukset: DNA-Diagnostiikan luotettavuus ja tarpeellisuus. XIX Tuusula Symposium. Gynekologiset Syövät. Tuusula, 6-7.2.1992.

4.Yliskoski, M., Hippeläinen, M., Kataja, V., Saarikoski, S., Syrjänen, K. Pregnancy and natural history of cervical HPV. 7th European Congress of Gynecology and Obstetrics, Helsinki, June 28.- July 1, 1992.

5.Yliskoski, M., Saarikoski, S. and Syrjänen, K. Conization for CIN associated with Human papillomavirus infection. Ob. Gyn. Digest. In press.

6.Chang, F., Syrjänen, S., Shen, Q., Wang, L., Wang, D. and Syrjänen, K. Human papillomavirus (HPV) involvement in esophageal precancerous lesions and squamous cell carcinoma as evidenced by microscopy and different DNA-techniques. Symposium on Human Tumorviruses, Heidelberg, Germany, May 3-6, 1992.

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8.Chang, F., Syrjänen, S., Wang, L. and Syrjänen, K. Preliminary results of the mass screening of human papillomavirus (HPV) infections in esophageal squamous cell carcinomas by in situ hybridization with biotinylated HPV DA probes in archieval specximens from the high-incidence area of China. 11th International Papillomavirus Workshop, Edinburgh, Scotland, September 5-11, 1992.

9.Chang, F., Syrjänen, S., Tervahauta, A., Kallio, P., Wang, L. and Syrjänen, K. Detection and characterization of DNA sequence homogeneous to human papillomavirus (HPV) genomes from esophageal squamous cell carcinomas. 11th International Papillomavirus Workshop, Edinburgh, Scotland, September 5-11, 1992.

10.Syrjänen, K. Epidemiology of HPV infections and genital neoplasia. First International Congress for Infectious Diseases in Obstetrics and Gynecology, Taormina-Giardini Naxos, June 14-19, 1992.

11.Syrjänen, K. Genital HPV infections in etiology of cervical cancer. XXth European Congress of Cytology, Prague, Czechoslovakia, September 23-26, 1992.

12.Syrjänen, S. and Syrjänen K. Papilloomavirusinfektio: Syövän aiheuttaja vai harmiton tulehdustauti? Dialogia Tiedotuslehti 3: 11-15, 1992.

13.Syrjänen, K.J. HPV typing: Does it help or not? Nordisk Förening för Ostetrik och Gynekologi, XXVIII Kongress. Reykjavik, Iceland, June 9-12, 1992.

14.Syrjänen, K. Virukset ja syöpä. Kirjassa: Suomen Syöpäinstituutin Säätiö, s. 16-17, 1992.

15.Ji, HX., Yliskoski, M., Väyrynen, M., Hippeläinen, M., Syrjänen, S., Syrjänen, K. Analisi colposcopica delle infezioni da papillomavirus genitale umano condotta con controlli prospettici durati otto anno. Giorn. It. Ost. Gin. 3, 216-224, 1992.

16.Syrjänen, K. HPV and CIN: Histopathological assessment. 7th Congress of the Italian Society of Colposcopy and Cervigo-Vaginal Pathology, Bologna, December 14-15, 1992.

17.Syrjänen, K. Risk factors in HPV-associated cervical carcinogenesis. 7th Congress of the Italian Society of Colposcopy and Cervigo-Vaginal Pathology, Bologna, December 14-15, 1992.

C) Doctoral (Ph.D.) Thesis

1.**Kellokoski J.** Human Papillomavirus (HPV) Infections of the Oral Cavity in Women with Genital HPV Infections. Publications of the University of Kuopio. Dental Sciences, Original Reports 1/1992.

2.**Kataja V.** Genital Human Papillomavirus (HPV) Infections. Prevalence, Incidence, Risk Factors and Prognosis. Kuopio University Publications. Medical Sciences, Original Reports 13/1992.

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REPORTS PUBLISHED FROM THE PAPILLOMAVIRUS PROJECT SINCE 1981

A.ORIGINAL ARTICLES, REVIEWS, BOOK CHAPTERS:

1.Syrjänen, K.J., Heinonen, U-M., and Kauraniemi, T. Cytological evidence of the association of condylomatous lesions with the dysplastic and neoplastic changes in uterine cervix. Acta Cytol. 25, 17-22, 1981.

2.Syrjänen, K.J. Epithelial lesions suggestive of a condylomatous origin found closely associated with invasive bronchial squamous cell carcinoma. Respiration 40, 150-160, 1980.

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6.Syrjänen, K.J. and Syrjänen, S.M. Histological evidence for the presence of condylomatous epithelial lesions in association with laryngeal squamous cell carcinoma. ORL, 43, 181-194, 1981.

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8.Syrjänen, K.J. Condylomatous lesions associated with precancerous changes and carcinomas of the uterine cervix. Neoplasma 28, 497-509, 1981.

9.Väyrynen, M., Romppanen, T., Koskela, E., Castren, O. and Syrjänen, K. Verrucous squamous cell carcinoma of the female genital tract. Report of three cases and survey of the literature. Int. J. Gynaecol. 19, 351-356, 1981.

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15.Syrjänen, K.J., Pyrhönen, S. and Syrjänen, S.M. Evidence suggesting Human papillomavirus (HPV) etiology for the squamous cell papilloma of the paranasal sinus. Arch. Geschwulstforsch. 53, 77-82, 1983.

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In any representation of the primary pathways responsible for the processing of sensory stimuli or motor inputs, it is notable that monoaminergic (noradrenergic, serotonergic, dopaminergic) neurons do not appear to be involved. The anatomical divergence of these latter neurons suggests that their networks are superimposed on those conveying sensory or motor signals. We have found some evidence that monoaminergic neurons, especially dopaminergic ones, favor the functional role of one or another cerebral structure, depending on the behavioural and external situations the animal is exposed to. These results seem important since dopaminergic neurons are extremely sensitive to most of the pharmacological products which induce addiction.

Amphetamine and cocaine increase the quantities of dopamine in the synaptic cleft. Moreover, dopaminergic neurons which innervate cortical and subcortical structures possess nicotinic receptors and are regulated by small interneurons which bear μ-opioid receptors. We have shown that repeated exposure to morphine increases the activity of dopaminergic neurons innervating *subcortical* structures. At the opposite, animals repeatedly exposed to nicotine demonstrate an activation of dopaminergic neurons innervating *cortical* structures, whereas dopaminergic neurons innervating *subcortical* structures remain unaffected.

Given that drug abuse necessarily involves repeated exposure to the drug and that such exposure to the nicotine was found to reduce its stimulatory effect on subcortical neurons, these results suggest that nicotine differs importantly from other abused drugs such as opiates and psychomotor stimulants. These latter are indeed generally accepted to achieve their rewarding properties through the activation of subcortical dopaminergic neurons.

Our data indicate that, in addition to subcortical structures such as the nucleus accumbens, prefrontal cortex may be a site involved in drug reward. Since dopaminergic neurons innervating cortical structures play a major role in cognitive processes, it can be proposed, therefore, that the activation of these neurons by nicotine represents the physiological basis of tobacco smoking's facilitatory effects on learning and increased attention. Such effects may be an important basis for the pursuit of nicotine.

J.P. TASSIN

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G_{olf} and G_s in Rat Basal Ganglia: Possible Involvement of G_{olf} in the Coupling of Dopamine D_1 Receptor with Adenylyl Cyclase

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Using specific antibodies and cDNA probes, we have investigated, in rat basal ganglia, the distribution and the regulation of the expression of the α subunits of G_s and G_{olf}, two GTP-binding proteins (G-proteins) that stimulate adenylyl cyclase. We confirmed that $G_{olf}\alpha$ is highly expressed in caudate-putamen, nucleus accumbens, and olfactory tubercle, whereas $G_s\alpha$ is less abundant in these areas than in the other brain regions. Intrastriatal injections of quinolinic acid decreased dramatically the levels of $G_{olf}\alpha$ protein in the striatum and the substantia nigra, and those of $G_{olf}\alpha$ mRNA in the striatum. Retrograde lesions of striatonigral neurons with volkensin reduced markedly the levels of D_1 dopamine (DA) binding sites, as well as those of $G_{olf}\alpha$ protein and mRNA in the striatum, without altering D_2 binding sites. In contrast, both types of lesions increased the levels of $G_s\alpha$ protein in the striatum and substantia nigra. Immunocytochemistry showed the presence of $G_{olf}\alpha$ protein in striatal medium-sized neurons and in several other neuronal populations. These results demonstrate that striatonigral neurons contain high levels of $G_{olf}\alpha$ and little, if any, $G_s\alpha$, suggesting that the coupling of D_1 receptor to adenylyl cyclase is provided by $G_{olf}\alpha$. The levels of $G_{olf}\alpha$ were five- to sixfold higher in the striatum than in the substantia nigra, indicating a preferential localization of $G_{olf}\alpha$ in the somatodendritic region of striatonigral neurons and providing a basis for the low efficiency of D_1 receptor coupling in the substantia nigra. Six weeks after 6-hydroxydopamine lesions of DA neurons, an increase in $G_{olf}\alpha$ (+53%) and $G_s\alpha$ (+64%) proteins was observed in the striatum. This increase in $G_{olf}\alpha$ levels may account for the DA-activated adenylyl cyclase supersensitivity, without change in D_1 receptors density, that follows destruction of DA neurons. Fine regulation of the levels of $G_{olf}\alpha$ in physiological or pathological situations may be a critical parameter for the efficiency of DA neurotransmission.

Key words: G-protein, $G_{olf}\alpha$, $G_s\alpha$, D_1 receptor, striatum, substantia nigra, basal ganglia, dopamine, signal transduction, adenylyl cyclase, receptor supersensitivity

Receptors with seven putative transmembrane domains are known to stimulate adenylyl cyclase via two homologous heterotrimeric G-proteins, G_s and G_{olf}, composed of α, β, and γ subunits and differing by their α subunits (Simon et al., 1991). Whereas G_s is found in numerous cell types (Gilman, 1987), G_{olf} is thought to mediate specifically the stimulation of type III adenylyl cyclase by odorant signal receptors in the olfactory neuroepithelium (Jones and Reed, 1989; Bakalyar and Reed, 1990; Menco et al., 1992). However, the recent demonstration of the presence of $G_{olf}\alpha$ mRNA in rat basal ganglia (Drinnan et al., 1991) suggests its possible involvement in the signal transduction cascade initiated by neurotransmitter-triggered receptors. One of the aims of the present study was to substantiate this possibility by studying the localization of $G_{olf}\alpha$ in striatonigral neurons, which express high levels of dopamine (DA) D_1 receptors (Gerfen et al., 1990; Harrison et al., 1990; Le Moine et al., 1991; Sibley and Monsma, 1992). OK.

Several lines of evidence suggest that the coupling efficiency of D_1 receptors with adenylyl cyclase could be a factor regulating the function of these receptors. In rat striatum, experiments with an irreversible D_1 blocker, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, have shown that 40% of D_1 receptors are "spare" receptors, not coupled to adenylyl cyclase, and have suggested that coupling could represent a limiting step in the D_1 receptor–dependent activation of adenylyl cyclase (Hess et al., 1987). Accordingly, long-lasting interruption of DA neurotransmission may increase the coupling efficiency of D_1 receptors in the striatum, since lesions of nigrostriatal DA neurons or chronic treatment with reserpine increases DA-stimulated adenylyl cyclase without changing the D_1 receptor density (Savasta et al., 1988; Hervé et al., 1989; Missale et al., 1989; Cowburn et al., 1991). The comparison of D_1 receptor densities and DA-sensitive adenylyl cyclase activities in several cerebral regions suggests also the existence of a regional variability in the coupling efficiency between these two proteins (Andersen et al., 1990). For instance, the DA-stimulated adenylyl cyclase activity is sevenfold higher in the striatum than in the substantia nigra, whereas the density of D_1 receptors labeled by ^{3}H-SCH23390 is similar in both structures (Hervé et al., 1992). Moreover, the density of D_1 receptors in high-affinity state for agonists, which is thought to correspond to the form associated with the G-protein (DeLean et al., 1980; Leff and Creese, 1985), is much lower in the substantia nigra than in the striatum (Hervé et al., 1992). These observations could be explained by variations in the levels of stimulatory G-proteins, which would be lower in the substantia nigra than in the striatum, and which would increase following long-lasting interruption of DA neurotransmission. In the present study, using cDNA and antibody probes specific for $G_s\alpha$ and $G_{olf}\alpha$, we demonstrate that $G_{olf}\alpha$ is enriched in striatonigral neurons, contrasting with $G_s\alpha$, which does not seem to be expressed in these neurons. We also show that $G_{olf}\alpha$ levels are

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EVIDENCE FOR A HIGH AFFINITY BINDING SITE FOR (-) SULPIRIDE IN THE RAT PREFRONTAL CORTEX: AN AUTORADIOGRAPHIC STUDY USING THE BENZAMIDE DERIVATIVE [^{125}I]-IODOSULPRIDE.

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Dimensions sociales de la demande de drogues 6-7-8 janvier 1993
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INTERETS DES RECHERCHES PHARMACOLOGIQUES POUR LA CLINIQUE

Jean-Pol Tassin (*)

L'étude de la toxicomanie représente l'occasion d'établir la base physiologique d'un comportement complexe correspondant à une réalité clinique. Plusieurs aspects de la conduite toxicomaniaque peuvent en effet, contrairement à d'autres troubles neuropsychiques tels que les désordres affectifs et psychotiques, être reproduits chez l'animal de laboratoire.

L'exposé de l'effet sur le cerveau des différents produits qui conduisent à une addiction passe par la compréhension du fonctionnement du neurone, et plus particulièrement du rôle d'un certain nombre de molécules, dites neuromédiateurs, qui jouent un rôle fondamental dans la transmission des flux ioniques dans les réseaux de neurones.

Le réseau de neurones qui constitue le système nerveux peut être assimilé à un réseau de communication extrêmement complexe. Certaines informations sont stockées, d'autres provoquent des communications en retour, d'autres enfin, transmises aux différents points de l'organisme, commandent le fonctionnement des muscles et des organes.

Le neurone et son neuromédiateur

Le neurone est la cellule nerveuse. Il se compose d'un corps cellulaire qui se prolonge de deux côtés : d'une part, il envoie des filaments dits dendrites, qui trouvent le contact d'autres neurones et captent des impulsions de nature électrochimique transmises par ces neurones; d'autrepart, il possède un long filament dit axone auquel le corps cellulaire transmet des impulsions. Ces impulsions, appelées potentiels d'action, parcourent l'axone et trouvent à son extrémité les dendrites d'autres neurones. L'arrivée du potentiel d'action à l'extrémité de l'axone entraîne la libération d'une molécule appelée neuromédiateur, qui se lie aux dendrites du neurone récepteur. Ainsi le neurone est un organe capable de recevoir des "informations" captées par ses dendrites, de les intégrer et de transmettre, par son axone, une "information" résultante aux dendrites d'autres neurones.

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→ European Journal of Neuroscience (1994)

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BLOCKADE OF PREFRONTO-CORTICAL α1-ADRENERGIC RECEPTORS PREVENTS LOCOMOTOR HYPERACTIVITY INDUCED BY SUBCORTICAL D-AMPHETAMINE INJECTION.

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NEUROANATOMIE FONCTIONNELLE ET LE PROBLEME DES RELATIONS STRUCTURE-FONCTION

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DNA ADDUCTS INDUCED BY ENVIRONMENTAL CHEMICALS

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Molecular events in chemical carcinogenesis involve the formation of DNA adducts of carcinogen metabolites but the relationship between this early event and later disease has not been fully elucidated. DNA adducts can be considered as molecular dosimeters for the assessment of the biologically effective dose resulting from genotoxic exposures.

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants and many of them are known carcinogens. Therefore, several studies have been carried out in occupational settings in which populations are exposed to complex mixtures of PAHs. Aromatic DNA adducts or PAH-DNA adducts have been investigated in white blood cells from coke-oven and iron foundry workers, roofers, firefighters, surface coating and aluminium plant workers. Adduct levels have been determined by ^{32}P-postlabelling, enzyme-linked immunosorbent assay, ultrasensitive radioimmunoassay and synchronous fluorescence spectrophotometry. Most of the studies have been cross-sectional and designed to investigate the relationship between exposure and DNA adduct levels. There have been large interindividual variations and in most cases enhanced levels of DNA adducts have been detected in exposed populations in comparison to controls. Dose-related adduct formation has been found in iron-foundry and coke-oven workers and roofers. A longitudinal study has been performed in two aluminium plants in Hungary on two occasions one year apart, in order to obtain information on temporal variability of exposure and its relationship to DNA adduct levels. A significant increase of adduct levels occurred in one of the plants from one year to the next which may have originated from a change of the workplace atmosphere at the period of the second sample collection.

More than 500 oil wells were set on fire in Kuwait in early 1991 which has produced a massive and unprecedented air pollution in the environment of the Persian Gulf region. Genotoxic effect of the chemically complex combustion material has been studied in white blood cell DNA of US personnel served in the region. Levels of aromatic DNA adducts determined by ^{32}P-postlabelling have not shown difference in a small group of individuals before and after the period of exposure. In a different investigation PAH-DNA adducts have been determined by dissociation-enhanced lanthanide fluoroimmunoassay before, during and after the period of exposure. Results have indicated elevated DNA adduct levels after the period of exposure in comparison to the previous two monitoring times.

DNA adducts are useful markers of genotoxic exposure on group basis. Recognition and quantitation of multiple adducts may vary with the methods applied. Therefore, adduct levels have to be considered relative between individuals and exposure groups. Investigation of DNA adducts may lead to a better understanding of mechanisms of chemical carcinogenesis and contribute to improved carcinogenic risk assessment.

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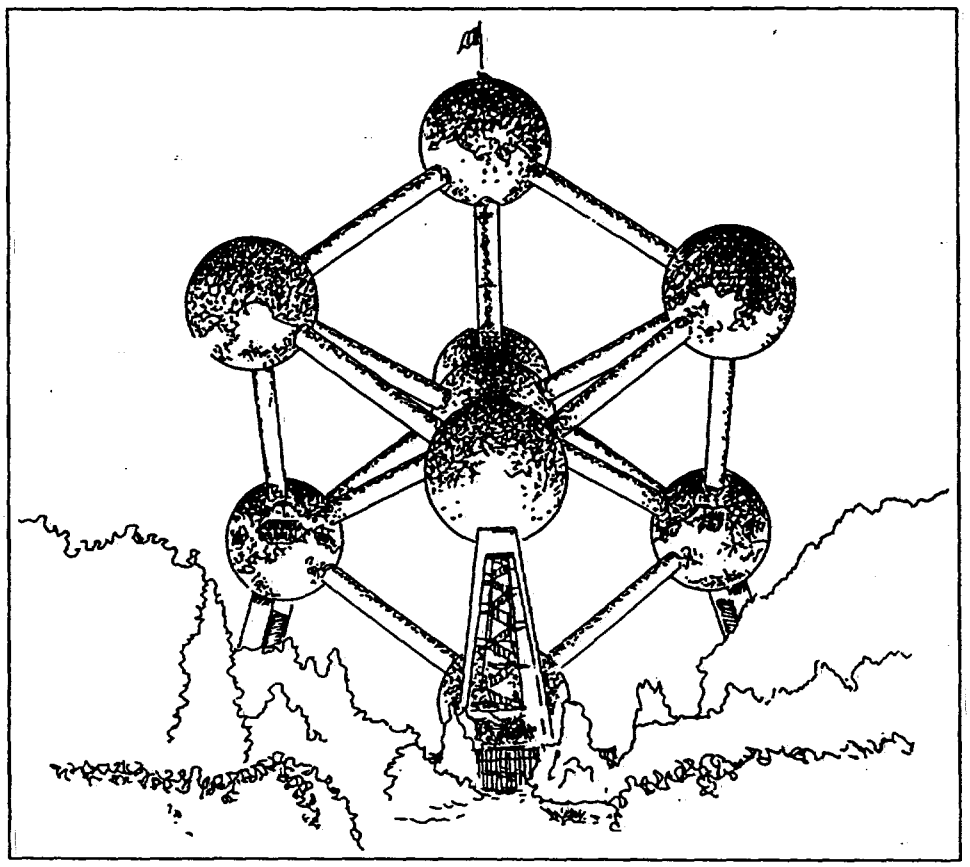

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EUROPE AGAINST CANCER

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SHORT COMMUNICATION

Smoking-related DNA adducts: ^{32}P-postlabeling analysis of 7-methylguanine in human bronchial and lymphocyte DNA

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7-methylguanine DNA adducts were determined in macroscopically normal bronchial specimens and peripheral blood lymphocytes of 20 patients undergoing pulmonary surgery. A recently developed ^{32}P-postlabeling assay was applied with anion exchange chromatography as an adduct enrichment method. The material consisted of 13 smokers and 7 non-smokers. The mean bronchial 7-methylguanine levels of 11 smokers and 6 non-smokers were 17.3 and 4.7 adducts/10^7 nucleotides. In lymphocyte DNA, the respective mean levels were 11.5 and 2.3 adducts/10^7 nucleotides. The bronchial DNA adduct levels in smokers were statistically higher than those in non-smokers. Among 5 smokers, for whom both bronchial and lymphocyte DNA was available, 7-methylguanine levels correlated in the two tissues (r = 0.77).

Tobacco is considered to be one of the major causes of cancer (1). Many of the several thousand chemicals present in tobacco smoke are carcinogenic (2). Many of these carcinogens are also known to form covalent DNA adducts (e.g. polycyclic aromatic hydrocarbons, aromatic amines, *N*-nitrosamines (3). The ^{32}P-postlabeling method has shown to be very suitable for detecting large, non-polar DNA adducts (4), and most human studies on tobacco-related DNA adducts, have focused on the polycyclic aromatic hydrocarbon-type of adducts (5–19). A potential role of tobacco-specific *N*-nitrosamines in smoking-related cancers has been proposed (20). The main tobacco-specific *N*-nitrosamines, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK) and *N'*-nitrosonornicotine (NNN), NNK being a methylating agent, are carcinogenic in animals, and smokers are estimated to be exposed to these compounds at significant levels (20). In a previous study the relationship between O^6-methylguanine in placental DNA and smoking was investigated (21). The present study is the first attempt to show by the ^{32}P-postlabeling assay 7-methylguanines in human target and non-target tissue DNA in respect to smoking. DNA adducts were measured in bronchial specimens and peripheral blood lymphocytes of smokers and non-smokers.

Sections of macroscopically normal bronchial tissue and blood were obtained from patients undergoing pulmonary surgery in the Thoracic Surgical Clinic of Postgraduation Medical School, Budapest, Hungary. Most of the 20 patients were carcinoma patients, except for patients no. 18 and 20 (Table I). Based on hospital records the patients were divided into smokers and non-smokers, i.e. never-smokers; the mean ages of these groups were 49.7 and 60.1 years respectively.

Lymphocytes were isolated by centrifugation in Ficoll Paque (14). DNA isolation from bronchial specimens and peripheral blood lymphocytes is described elsewhere (7). The procedure for DNA digestion, adduct enrichment with anion exchange chromatography and ^{32}P-postlabeling of human DNA (13–19 μg) is described elsewhere (22,23). In the present study the labeled samples were applied to 10 × 10 cm TLC plates (Maherey Nagel) and developed in the first dimension with 0.1 M ammonium format, pH 5.3, and in the second dimension with 0.1 M LiCl (Figure 1). Student's *t*-test was used for statistical evaluations as the data were normally distributed. Different tests were applied when variances in the data sets were equal or nonequal.

Figure 2 shows chromatographic patterns of 7-methylguanine analyses of bronchial and lymphocyte DNA isolated from smokers and non-smokers. Individual 7-methylguanine adduct determinations are based usually on 3 separate ^{32}P-postlabeling analyses. The mean bronchial adduct levels in 11 smokers and 6 non-smokers were 17.3, and 4.7 7-methylguanines/10^7 nucleotides respectively (Figure 3). The corresponding values in lymphocyte DNA were 11.5 and 2.3 7-methylguanines/10^7 nucleotides respectively, in 7 smokers and 3 non-smokers. Statistical analysis showed that the mean bronchial adduct levels of smokers were significantly higher than the means of non-smokers. In lymphocyte DNA the difference between smokers and non-smokers was of borderline significance ($P = 0.055$). When 7-methylguanine levels in bronchial and lymphocyte DNA, obtained from the same smokers, were compared, a correlation was observed (Figure 4). Unfortunately, the correlation (r = 0.77) was based on only 5 individuals from whom both tissue samples were available (cf. Table I). There was no correlation in bronchial tissue or in lymphocytes between the adduct levels and the daily consumption of cigarettes (data not shown).

Several reports have demonstrated, by using mainly the ^{32}P-postlabeling assay, the presence of aromatic adducts in smokers' bronchus or lung tissue (5–21). However, tobacco smoke contains more than 4 different compounds suggested to be carcinogenic (24). Among these, the main tobacco-specific *N*-nitrosamines, NNK and NNN, are both found in relatively high levels in mainstream and sidestream smoke and also in unburned tobacco (20). A potential role of NNK and NNN in the induction of cancers of the lung and esophagus has been proposed. Hydroxylation reactions are suggested as the main metabolic pathways for tobacco-specific *N*-nitrosamines, resulting at least in the case of NNK in the formation of methyl diazonium hydroxide, capable of DNA methylation (20).

DNA methylation at the O^6- and N-7 positions of guanine by tobacco-specific *N*-nitrosamines has mainly been identified in the target tissue of experimental animals (25,26). A correlation between O^6-methylguanine levels, suggested promutagenic

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Table I. Descriptive data of the patients whose bronchial and blood samples were analysed

Patient no[a]	Age Time since stopping smoking[c]	Diagnosis[b]	Years smoked	Smoking history Cigarettes/day
Smokers				
1 (m)	62	S	42	10
2 (m)	53	S	35	3
3 (m)	48	S	33	20
4 (m)	47	A	32	8
5 (m)	67	A	50	20
6 (m)	64	AN	50	60
7 (m)	38	S	20	25
8 (m)	58	S	45	20
9 (f)	51	SC	32	20
10 (f)	39	L	33	10
11 (f)	42	A	20	6
12 (f)	40	AN	26	16
13 (f)	37	A	12	7
Non-smokers				
14 (m)	65	A		
15 (m)	69	S		
16 (f)	59	A		
17 (f)	53	A		
18 (f)	64	pneumonitis		
19 (f)	66	A		
20 (f)	45	cystadenoma		

[a]m = male; f = female.
[b]A, adenocarcinoma; AN, anaplastic carcinoma; L, large cell carcinoma; SC, small cell carcinoma; S, squamous cell carcinoma.

adducts (27), and the formation of pulmonary neoplasia has been shown in rats (28). In a few studies methylated DNA adducts (O^6-methylguanines) have been analysed in human DNAs from different sources (21,29–31). In the present study we analysed 7-methylguanines in human DNA by using the ^{32}P-postlabeling assay with a recently developed anion exchange chromatography as an adduct enrichment method (22,23). Patients undergoing pulmonary surgery offered an opportunity to compare the presence of DNA adducts in bronchial tissue and peripheral blood lymphocytes in relation to smoking.

The present data demonstrated statistically significant differences between smokers' and non-smokers' bronchial DNA: smokers had ~5-fold higher mean adduct levels than non-smokers respectively. In the present study, the mean adduct level in smokers as compared to non-smokers was almost significant in lymphocytes. Our recent results with healthy volunteers also showed a difference between smokers and non-smokers (23).

Interindividual variations in the level of adducts were large, 27–46-fold, in bronchial DNA of smokers and non-smokers respectively; in the lymphocyte DNA the adduct levels varied somewhat less. The adduct values obtained here are in general in the range of our previous results for white blood cells (22,23). Shields *et al.* (32) reported, using combined high-performance liquid chromatography/^{32}P-postlabeling, levels of 7-methylguanines comparable to the present bronchial levels in lung samples of 5 trauma victims, who were smokers.

The adduct levels in bronchial tissue of the individual smokers correlated with the lymphocyte adduct levels (r = 0.77). This is an important observation, although based on 5 individuals only, because it shows for the first time that a specific adduct in lymphocytes is correlated with the adduct level in the target tissue. In a previous study on smokers van Schooten *et al.* (19) reported no correlation between total white blood cell and lung adducts,

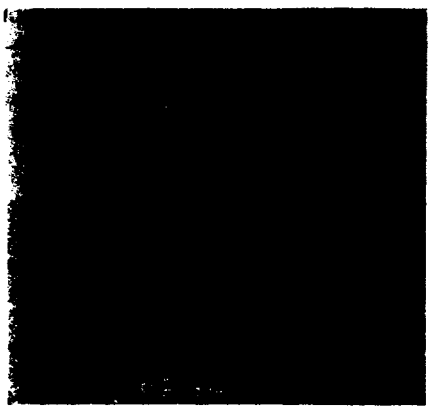

Fig. 1. An autoradiogram of a polyethylene imine-cellulose TLC plate of 7-methylguanines enriched by anion exchange chromatography. DNA (13–19 μg) isolated from bronchial specimens or peripheral blood lymphocytes were labeled with [γ-^{32}P]-in the presence of T4 polynucleotide kinase for 1 h after which 3'-phosphates were removed with nuclease P1. 7-Methylguanines were separated in a two-dimensional thin-layer chromatography system using 0.1 M ammonium formate, pH 5.3, in the first direction. The plate was cut, as shown, before development of the second direction with 0.1 M LiCl. The exposure time of autoradiographic X-ray films (Kodak XAR-5) was 1–3 hours. For details of the procedure see (22,23).

but in fact the correlation among 8 current smokers was reasonable (r = 0.52). The analysis was based on no specific smoking-induced adducts.

We found no correlation between the bronchial nor lymphocyte adduct levels and the daily consumption of cigarettes. Several possible factors may modulate the individual levels of 7-methylguanines. Large interindividual differences in many

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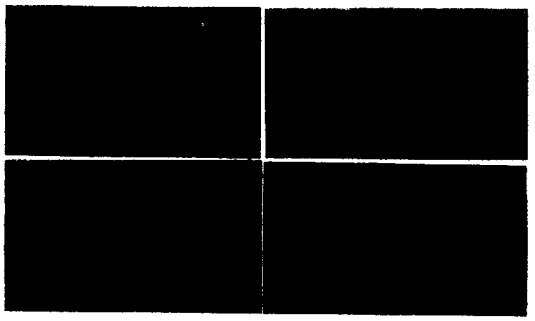

Fig. 2. The autoradiograms of polyethyleneimine-cellulose maps of 7-methylguanine adducts ^{32}P-postlabeled from bronchial (B) or blood lymphocyte (L) DNA of a non-smoker (1) and a smoker (4).

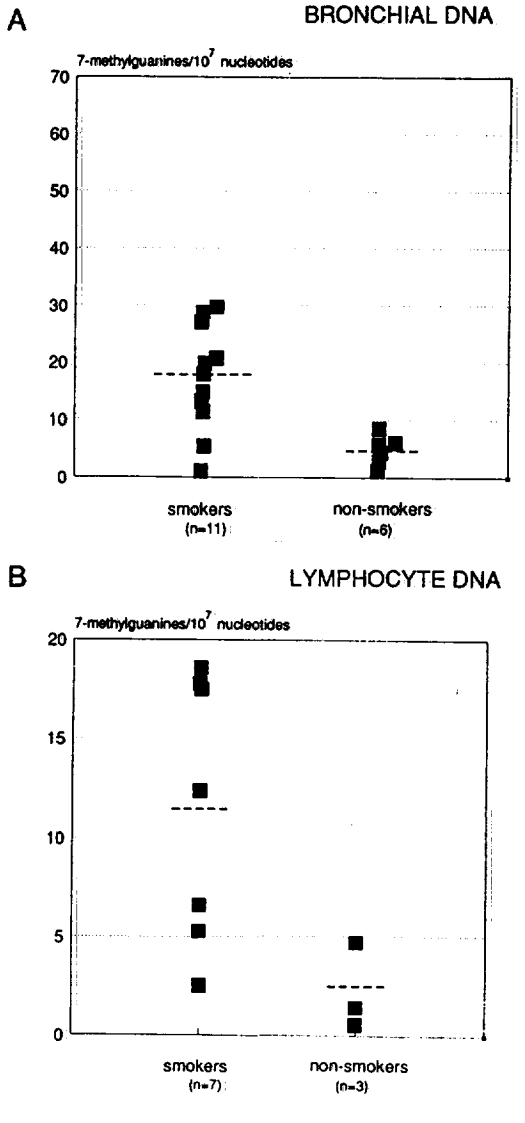

Fig. 3. Individual 7-methylguanine levels in bronchial (A) and lymphocyte (B) DNA of smokers and non-smokers. The differences between smokers and non-smokers were significant at $P < 0.01$ in bronchial samples and at $P = 0.055$ in lymphocytes.

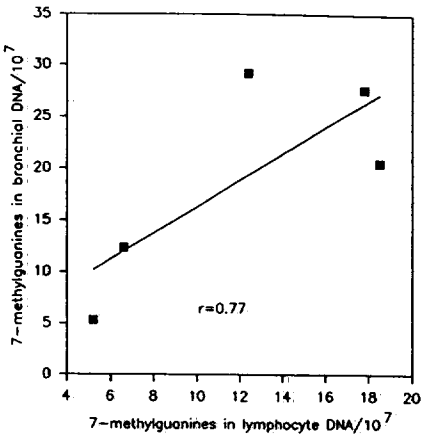

Fig. 4. Correlation of 7-methylguanine levels between bronchial and lymphocyte DNA from smokers.

pulmonary drug-metabolizing enzyme activities have been reported in humans (33,34). In tissue cultures and experimental animals the metabolism of different tobacco-specific *N*-nitrosamines and repair of methylated DNA demonstrated tissue and cell type specificity (35–37). Also little is known about spontaneous depurination *in vivo* and how it might vary between individuals. Lastly, the extent of endogenous methylation by e.g. S-adenosylmethionine and the formation of *N*-nitroso-compounds may also be confounding factors. Food is known to contain both precursors as well as catalysts and inhibitors of *in vivo* nitrosation (38).

In summary, the present study demonstrated the presence of 7-methylguanines in human target and non-target DNA in relation to smoking. In a small group of current smokers the levels of 7-methylguanine correlated between bronchial and lymphocyte DNA.

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Abstract submitted for the Postlabelling Meeting in Lyon, June 199

DETERMINATION OF SMOKING-RELATED DNA ADDUCTS IN LUNG-CANCER AND NON-CANCER PATIENTS

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Molecular events of tobacco-associated carcinogenesis involve the formation of DNA adducts of carcinogen metabolites which is considered a necessary early step in this adverse process. DNA has been isolated from uninvolved bronchial tissues of 98 patients undergoing pulmonary surgery and aromatic DNA adduct levels have been determined by ^{32}P-postlabelling using the Nuclease P1 adduct enrichment. Correlations have been investigated between levels of DNA adducts, smoking status, histological diagnosis and other factors. Statistical evaluations have been made by Mann-Whitney U-test. Mean DNA adduct level of the 45 current smokers was 9.87 ± 4.06 adducts in 10^8 nucleotides which was significantly higher ($p=0.022$) than that of the 16 life-time non-smokers having 7.59 ± 3.63 adducts in 10^8 nucleotides. DNA adduct levels of those 25 former smokers, who stopped smoking within a year before surgery, did not differ significantly from the current smokers' adduct level. Those 12 former smokers, however, who had given up smoking more than a year before surgery showed decreased levels of DNA adducts which was the same as life-time non-smokers'. Non-cancer patients, individuals with squamous-cell carcinoma and with other lung cancers exhibited very similar adduct levels, and no significant difference of adduct levels was found if cancer and non-cancer patients were compared either among the current smokers or life-time non-smokers. The results have given information on the persistence and slow repair of smoking-related aromatic DNA adducts in human bronchial tissue. Simple quantitative association has not been found between bronchial DNA adduct level and lung cancer.

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Original

Determination of smoking-related DNA adducts in human tissues

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Molecular events of tobacco-associated carcinogenesis involve the formation of covalent DNA addition products (DNA adducts) of carcinogen metabolites which is considered a necessary early step in this adverse process. The ^{32}P-postlabelling technique was used for the determination of bulky aromatic adducts in uninvolved bronchial tissues and peripheral blood lymphocytes from one hundred patients undergoing lung surgery. Smokers exhibited a significantly higher level of bronchial DNA adducts than non-smokers. Bronchial DNA adduct levels of former smokers who stopped smoking less than a year ago did not differ significantly from those of current smokers which indicates the persistence and slow repair of this type of DNA damage. Small alkylated adducts are partly repaired by the O^6-alkylguanine alkyltransferase. Activity of this enzyme was measured in lung tissues of the same patients and it was elevated in smokers. Levels of DNA adducts and activity of O^6-alkylguanine alkyltransferase were not significantly different between the cancer and non-cancer groups of patients. Genotoxic effect of smoking was clearly shown but direct quantitative association between the investigated markers and manifestation of lung cancer was not found.

Abstract submitted for the 2nd annual congress of European Respiratory Society in Vienna, Sept 1992

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Organizations, etc

1976 -	Member of the Nobel Assembly, Karolinska Institute and subcommittees
1993-	Vice chairman, Nobel Assembly, Karolinska Institute
1978 - 1985	Member of the Executive Board, and faculty subcommittees, Karolinska Institute
1980 - 1986	Member of the Swedish Medical Research Council, Committee for Physiology
1980 - 1984	Councillor, European Association for the Study of Diabetes
1981 - 1987	Honorary Treasurer and Secretary, European Society for Parenteral and Enteral Nutrition
1979 - 1985	Editor-in-Chief, Clinical Physiology
1985 -	Associate Editor, Clinical Physiology
1980 - 1983	Associate Editor, Diabetologia
1983 - 1985	Associate Editor, Nutrition International

Scientific awards

1976	Alvarenga Award: Swedish Medical Society
1979	Minkowski Award: European Society for the Study of Diabetes
1993	Henry Christian Award for excellence in science, American Federation for Clinical Research

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John Wahren

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1982 - 1993

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John Wahren

Nicotine Physiology and Metabolism

Summary of current projects

1. The current status of knowledge regarding metabolism and pharmacokinetics of nicotine was updated and summarized at an international symposium in June 1992 at Salsomaggiore, Italy. The meeting was organized by Professors Gorrod and Wahren, who will also be the editors of the proceedings from the meeting. The publication will be a monograph entitled "Biochemistry and Metabolism of Nicotine and Related Alkaloids", published by Chapman and Hall Scientific Publishers, London. The work with the monograph is now in its final stages and publication is expected in July or August, 1993.

2. Studies are in progress concerning the influence of nicotine on regional circulation and metabolism of the human brain. They involve "functional imaging" of the brain by NMR-technique as well as determination of regional brain blood flow by PET-technique. The studies are being done in collaboration between the Karolinska Hospital, Stockholm and the Max-Planck Institute in Göttingen. The preliminary findings in three individuals receiving intravenous nicotine indicate that blood flow to the visual and auditory cortex is augmented by nicotine and, conversely, that flow to the cerebellum is diminished. The NMR- and PET-techniques lend themselves excellently to this type of study and the results may be the first demonstration of regional effects of nicotine on brain blood flow and metabolism in humans.

3. A study concerning hepatic and renal nicotine disposal in humans has been carried out together with Johan Gabrielsson. The results provide the first direct demonstration and quantitataive measurement of hepatic nicotine extraction in humans. The data were presented in preliminary form at the Salsomaggiore meeting and have now been evaluated and written up for a peer review journal. The final manuscript is now being adjusted and will be submitted within a month. It is a project that has received FTR support.

4. The effect of cigarette smoking on whole body energy expenditure on a short-term basis has been examined using indirect calorimetry. These studies extend previous findings concerning cigarette smoking and 24 h energy expenditure, published earlier by Hofstetter and Wahren in New England Journal of Medicine. The data describe the magnitude, time course and mechanism of nicotine's stimulatory action on energy expenditure. The study has been supported by FTR. A manuscript is under way and should be ready for submission by the end of June, 1993.

5. A study is in progress concerning the possible influence of liver disease on nicotine disposal. We have examined patients with cirrhosis of the liver during nicotine infusion. Additional studies are required, but the preliminary data indicate a delayed disposal of nicotine and a retarded conversion of nicotine to cotinine. A completed study should be ready within 4-6

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months depending on the availability and outcome of the nicotine analyses.

6. A study has been undertaken to examine the relationship between arterial nicotine concentrations during cigarette smoking and nicotine content and puffing pattern. Thirty subjects participated in the study and a pneumotachograph was employed to determine the puff characteristics. The findings demonstrate large intra-individual variations in arterial nicotine concentration during smoking and that the cigarettes' nicotine yield or the smoking pattern both are of minor importance for the arterial nicotine concentration. The data have been evaluated and a preliminary manuscript is ready. The final paper will be sent for publication within 1-2 months.

Publications in progress

1. Biochemistry and metabolism of nicotine and related alkaloids, eds John Gorrod and John Wahren, Chapman and Hall, London, 1993 in press.

2. J Wahren. New methods for probing the *in vivo* disposition of nicotine in humans, in Biochemistry and metabolism of nicotine and related alkaloids, eds J Gorrod and J Wahren, Chapman and Hall, London, 1993 in press.

3, J Wahren, J Gabrielsson, M Curvall. Hepatic and renal exchange of nicotine in humans. J Clin Pharmacol Ther 1993, to be submitted.

4. J Wahren, Y Schutz, R Söderberg. Stimulation of energy expenditure during cigarette smoking. Am J Physiol 1993, to be submitted.

5. J Wahren, R Söderberg. Arterial nicotine and cotinine concentrations and cigarette smoking patterns. Clin Sci 1993, to be submitted.

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